UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

GRADUATE EDUCATION
(301) 295-3913
FAX: (301) 295-6772

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Name of Student: LT John D. Schaber

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DECISION OF EXAMINATION COMMITTEE MEMBERS:

	PASS	FAIL
Richard Conran, Ph.D., MD Department of Pathology Chairperson	✓	
Hallgeir Rui, Ph.D. Department of Major Advisor	✓	
Robert Friedman, ~~Ph.D.~~ MD Department of Pathology Member	✓	
Val G. Hemming, M.D. Department of Pediatrics Member	✓	
Peter Soballe, M.D. Department of Surgery Member	✓	

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LT John D. Schaber
Department of Pathology
Uniformed Services University of the Health Sciences

ABSTRACT

Title of Dissertation: Expression and Activation of STAT Transcription Factors in Breast Cancer

John D. Schaber, Doctor of Philosophy, 1998

Dissertation directed by: Hallgeir Rui, M.D., Ph.D., Assistant Professor, Department of Pathology

Endocrine therapy has proven a valuable approach to the treatment of breast cancer. In particular, antiestrogens have demonstrated significant improvement in survival rates, and have recently been shown to prevent breast cancer development in women in high-risk populations. Other endocrine or cytokine-based therapies, including glucocorticoids and interferons, which have been highly effective as adjuvant treatment of hematological cancers, have also shown promise in breast cancer. However, due to the less consistent clinical responses to both glucocorticoids and interferons in breast cancer patients, research efforts have continued to focus on improving their efficiency.

Important recent insights into the underlying molecular biology of hormone signal transduction have identified the

critical involvement of cytoplasmic STAT transcription factors. These molecular intermediaries convey the signal from the cell surface to the nucleus where they activate transcription of target genes. One hormone, which signals via the STAT pathway, is of particular relevance in breast cancer: namely, the mammary growth and differentiation factor, prolactin.

The specific aims of this study were: 1) to examine whether the glucocorticoid, dexamethasone, may promote the terminal differentiation of breast cancer cells by stimulating prolactin activation of the transcription factors, STAT5a and STAT5b; 2) to examine whether prolactin interferes with type I interferon signal transduction by competing for limited cytoplasmic STAT factors, thus antagonizing the antiproliferative effect of type I interferons in breast cancer treatment; and 3) to test if mammary tumor cell lines, like many hematopoietic cancer cells, become sensitized to the anti-proliferative effect of type I interferons by pretreatment with interferon-gamma.

After establishing differentiation conditions in breast cancer cells, STAT transcription factor expression, activation and DNA-binding were examined by immunoblot and electrophoretic mobility shift assay. Based on the research work presented in this thesis, we conclude that:

1) Glucocorticoids have a profound positive effect on prolactin signal transduction by STAT5 transcription factors in some, but not all breast cancer cells. STAT5a expression

is clearly linked to differentiation of breast cancer cells, and our findings may have important implications for the use of glucocorticoids in differentiation therapy of select breast cancer patients.

2) Prolactin activates STAT1 but does not disrupt STAT1-STAT2 heterodimer formation or the anti-proliferative effect of type I interferons in human breast cancer cells. In fact, cytoplasmic levels of STAT1 are not rate-limiting, and prolactin and type I interferons maintain an unexpectedly high degree of signal fidelity in human breast cancer cell lines despite activating overlapping sets of STAT transcription factors.

3) Pretreatment of mammary cancer cells with interferon-gamma enhanced signal transduction and antiproliferative effect of type I interferons (alpha/beta), a finding that may lead to improved interferon-based therapy of breast cancer patients.

EXPRESSION AND ACTIVATION OF STAT TRANSCRIPTION FACTORS IN BREAST CANCER

by

JOHN DAVID SCHABER

Dissertation submitted to the Faculty of the Department of Pathology Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for degree of Doctor of Philosophy

1998

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Institute of Molecular Medicine and Genetics
Medical College of Georgia

Hallgeir Rui, M.D., Ph.D.
Department of Pathology
Uniformed Services University of the Health Sciences

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TABLE OF ABBREVIATIONS

α-	anti- (eg., α-Stat5a = anti-Stat5a)
ATCC	American Type Culture Collection
BCPT	Breast Cancer Prevention Trial
BSA	bovine serum albumin
CNBr	cyanogen bromide
CSF	colony stimulating factor
DBD	DNA binding domain
DEX	dexamethasone
DMSO	dimethyl sulfoxide
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetate
EGF	epidermal growth factor
EMSA	electrophoretic mobility shift assay
EPO	erythropoietin
ER	estrogen receptor
ERK	extracellular-regulated kinase
FGF	fibroblast growth factor
GAS	gamma activated sequence
GRR	gamma response region

HEPES	N-hydroxyethylpiperazine-N′-2-ethanesulfonic acid
HPL	human placental lactogen
HTLV	human T-cell leukemia virus
IFN	interferon
IGF	insulin-like growth factor
IL	interleukin
INS	insulin
IP	immunoprecipitate
IRF1	interferon regulatory factor-1
ISG15	interferon-stimulated gene 15
ISRE	interferon stimulation response element
JAK	Janus kinase
JH	JAK homology (domain)
kDa	kilodalton
LIF	leukemia inhibiting factor
MAPK	mitogen-activated protein kinase
MGF	mammary gland factor
MTT	dimethylthiazolyl diphenyl tetrazolium bromide
OSM	oncostatin M
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PIP	prolactin-inducible protein

PMSF	phenylmethylsulfonyl fluoride
PRL	prolactin
PVDF	polyvinylidene diflouride
PY	phosphotyrosine
SDS	sodium dodecylsulfate
SH2	src homology 2
SSC	saline-sodium citrate
STAT	signal transducer and activator of transcription
TEMED	tetramethylethylene diamine
trans	transactivating

INTRODUCTION

Breast cancer is a major cause of morbidity and mortality in the United States. Each year, more than 180,000 women are diagnosed with invasive carcinoma, and over 40,000 succumb to the disease. Breast cancer therefore ranks as the most common malignancy and the second leading cause of cancer death among women in the United States (Kopans, 1998).

Although the incidence of breast cancer has risen nominally over the past ten years, this increase has been offset in large part by improvements in early detection and treatment, leaving the overall mortality rate essentially unchanged (Fisher, et al., 1996). That is, as the proportion of patients with minimal disease increases, so too increases the percentage of patients for whom surgery (with or without radiation) is curative.

Unfortunately, the value of early detection has practical limits, and many patients will continue to develop more advanced disease. Indeed, for most patients, breast cancer is now believed to be a systemic disease at the time of detection (Fisher, et al., 1996). Thus, the need for a better understanding of the fundamental molecular biology of

mammary carcinogenesis and tumor progression has not diminished.

Breast cancer arises from the dysregulation of normal growth and differentiation patterns in mammary epithelial cells. This tissue is remarkable in that it is responsive to an extraordinarily large number of hormones and cytokines whose often-conflicting signals must be carefully integrated to serve the demanding biological function of the mammary gland. That function requires not only that the mammary epithelium undergo massive proliferation and differentiation during pregnancy, but that it also involute to the prepregnant state following weaning. Further, even during periods of nonpregnancy, the mammary epithelium undergoes a monthly cycle of proliferation and regression during menses.

Because of this highly proliferative character, the mammary epithelium is especially prone to accumulate mutations. An above-normal rate of cellular proliferation can enhance the transforming effect of carcinogens by propagating isolated mutations (Kinzler, et al., 1996). This will have an increasingly negative impact on tumor suppressor genes and DNA-repair mechanisms, leading to the progressive genetic instability that characterizes transformed cells (Klein, et al., 1990). It is not surprizing, then, that such a

complexly-regulated, mutation-prone system is also particularly susceptible to neoplastic transformation, making adenocarcinoma of the breast the most common malignancy in women (Fisher, et al., 1996).

To better understand mammary carcinogenesis, we must first consider the underlying physiology of the breast.

ENDOCRINE PHYSIOLOGY OF THE BREAST

During the reproductive years, the breast undergoes a cycle of proliferation and involution in response to hormonal influences of the menstrual cycle in preparation for pregnancy. The following briefly recapitulates the hormonal changes associated with the menstrual cycle and pregnancy.

Menstrual cycle changes

In the proliferative phase of the menstrual cycle, rising estrogen levels - in concert with prolactin, insulin, insulin-like growth factor 1 (IGF-1), epidermal growth factor (EGF), glucocorticoids, and growth hormone - induce lobuloalveolar development with the formation of epithelial sprouts and alveoli (Longacre, et al., 1986). Increasing progesterone levels in the luteal phase are associated with

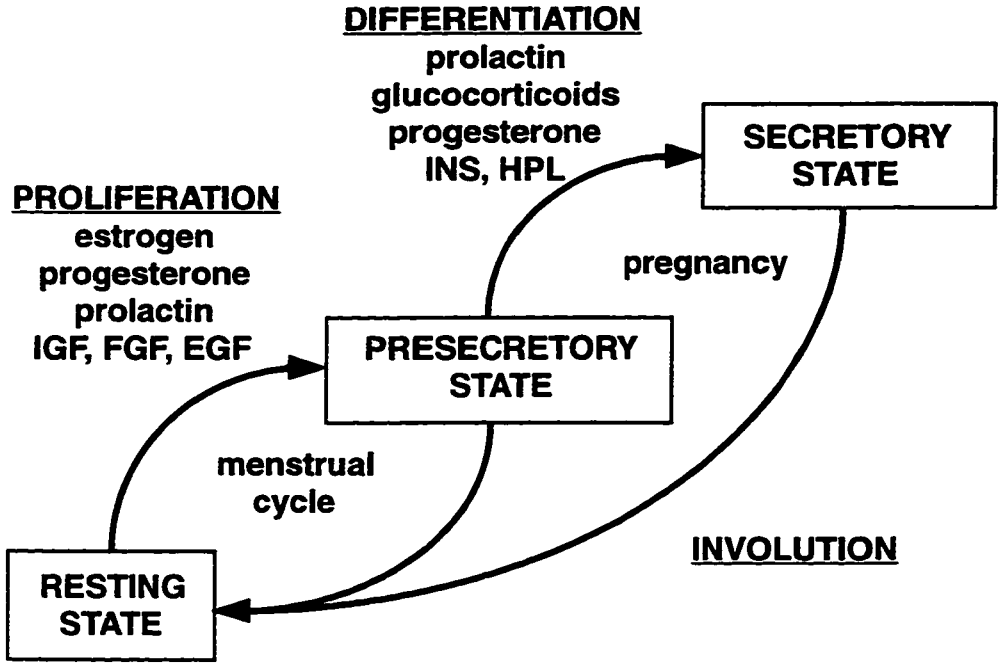
dilatation of the ductal system and partial differentiation of the alveolar cells into presecretory units (see fig. 1). Late in cycle, up-regulated prolactin secretion and declining steroid hormone levels cause modest secretory activity (Fanager, et al., 1974). Interestingly, although estrogen and progesterone are generally regarded as the principal sex steroids involved in breast growth and development, the requirement of prolactin is absolute (Dickson, et al., 1995).

At the end of the menstrual cycle, withdrawal of growth factor and steroid hormone support leads to significant mammary involution. Mediated by apoptotic processes, this regression is characterized by a decrease in stromal cellularity and a reduction in the ductal and alveolar epithelial component (Longacre, et al., 1986).

Pregnancy

During pregnancy, the mammary epithelium undergoes an even more marked proliferation through the complex interactions of a number of ovarian, pituitary, adrenal, and placental hormones. During the first trimester, increasing levels of steroid hormones in the maternal circulation, particularly estrogen, promote further elaboration of the lobuloalveolar system. The inhibitory effects of estrogen and

Fig. 1. Endocrine physiology of the breast during the reproductive years. In the proliferative phase of the menstrual cycle, estrogen drives mammary epithelial proliferation, in concert with progesterone, prolactin, glucocorticoids, and other factors. Withdrawal of hormone support at menses leads to mammary involution mediated by apoptotic processes. During pregnancy, the breast undergoes tremendous proliferation, again driven principally by estrogen. At parturition, estrogen and progesterone levels decline and the mammary epithelium becomes fully differentiated in response to prolactin. Upon weaning, hormonal support is withdrawn and the breast involutes to the prepregnant state.


DIFFERENTIATION
prolactin
glucocorticoids
progesterone
INS, HPL
SECRETORY STATE
PROLIFERATION
estrogen
progesterone
prolactin
IGF, FGF, EGF
pregnancy
PRESECRETORY STATE
menstrual cycle
INVOLUTION
RESTING STATE

progesterone upon the hypothalamic release of the prolactin inhibiting factor, dopamine, also cause prolactin to rise progressively throughout gestation (Reyniak, 1979). Indeed, as noted above, prolactin is required for full expression of the mammotropic effects of estrogen and progesterone (Dickson, et al., 1995). Toward the end of pregnancy, the breast reaches full development in preparation for lactation. Although prolactin levels continue to rise, approaching levels tenfold above the prepregnant state, the full differentiation effects of prolactin are inhibited by the sex steroids (Reyniak, 1979). Only after parturition, when estrogen and progesterone levels begin to drop, does the mammary epithelium become fully differentiated and begin to express milk.

Sustained nursing maintains the mammary epithelium in the fully differentiated state through continued pituitary prolactin secretion. Upon weaning, however, this hormonal support is withdrawn and the gland undergoes massive involution to the prepregnant state, as described above.

PROLACTIN IN MAMMARY PHYSIOLOGY

As outlined above, the role of prolactin in mammary

growth and development is critical. In recent years, great advances have been made in understanding the molecular details of prolactin signal transduction. Despite decades of research, however, many of the fundamental questions about the biology of prolactin remain unanswered.

Mitogen or differentiation factor?

There is evidence that prolactin can serve either as a mammary epithelium growth promoter on the one hand, or as a cytostatic, terminal differentiation agent on the other (Shiu, et al., 1984; Rosen, et al., 1994; Fuh, et al., 1995). In conjunction with estrogen and progesterone, prolactin acts as a growth factor and is absolutely required for mammary growth and development. In the presence of glucocorticoid hormones, however, prolactin operates as a differentiation factor, promoting lactogenesis (Dickson, et al., 1995). Indeed, steroid hormones of the glucocorticoid family (ie., cortisol) are required for prolactin to cause the final differentiation of the alveolar epithelial cells into mature milk cells (Topper, et al., 1980; Merlo, et al., 1996). Other stromal factors and the extracellular matrix itself also contribute to prolactin-induced mammary differentiation (Roskelley, et al., 1994).

There is, thus, compelling evidence suggesting that prolactin is a conditional modulator of mammary growth (Doppler, 1994). The dual capacity of prolactin to induce either cellular proliferation or differentiation of mammary target cells, suggests the presence of multiple physiological regulatory mechanisms that can control response switching at the cellular level. Implicit in this notion that prolactin-induced responses are dependent on physiological conditions is the concept that prolactin might function as tumor promoter only when the physiological environment or specific pathophysiologic changes favor prolactin receptor-mediated growth. Indeed, the prolonged controversy over the involvement of prolactin in human breast and prostate cancer etiology and progression might be resolved if circulating or autocrine prolactin (Ginsburg, et al., 1995) were proven to serve as a conditional tumor promoter.

A duality of prolactin actions might also provide an explanation as to why multiparity and prolonged nursing tend to lower breast cancer risk. It would be reasonable to hypothesize that perinatal physiological conditions might yield a protective effect by fostering the differentiating effects of prolactin. Thus, a better understanding of how prolactin acts as a conditional growth factor or tumor

promoter, or conversely as a differentiation agent, becomes a critical issue with strong relevance to the problem of growth factor-induced breast cancer development and growth factor-based therapeutic strategies.

Prolactin and cancer:

Although several lines of evidence point to prolactin as a permissive risk factor for human breast cancer (Malarkey, et al., 1983), the extent of prolactin involvement in the etiology of human breast carcinogenesis in particular, and tumor progression in general, has remained unresolved and controversial. Overstimulation of prolactin receptors in experimental rodent models has been established as a mechanism of prostate, liver and breast tumor promotion (Nakamura, et al., 1990; Buckley, et al., 1985; Welsh, et al., 1977; Tejwani, et al., 1991). In fact, 40-70% of human breast tumor biopsies are positive for prolactin receptors (Bernstein, et al., 1993; Bonneterre, et al., 1990). Similarly, many breast cancer cell lines express increased levels of prolactin receptors and can proliferate in response to prolactin in vitro (Shui, 1979; Shui, et al., 1985; Manni, et al., 1986; Malarkey, et al., 1983). Intriguing anecdotal cases of human breast cancers related to prolactin have

included males with prolactinoma (Bernstein, et al., 1993).

On the other hand, there is significant, unexplained evidence against a carcinogenic role for prolactin. One such argument points to the tenuous evidence for any correlation between elevated levels of circulating prolactin and an increased risk for breast cancer (Zumoff, et al., 1994; Ingram, et al., 1990; Love, et al., 1991; Wang, et al., 1992). Indeed, it is noted that multiple pregnancies and prolonged nursing, which should expose parous women to increased prolactin levels, are statistically risk mitigating (Kalache, et al., 1993). A second counter-argument has been the disappointing results of clinical trials testing drugs that block pituitary prolactin secretion as anti-hormonal adjuvant therapy (Peyrat, et al., 1994).

More recently, investigators have detected local production of prolactin in mammary epithelium and human breast carcinomas, indicating that prolactin may act as an autocrine mammary growth factor (Fields, et al., 1993; Clevenger, et al., 1995; Ginsburg, et al., 1995). Consistent with a local growth stimulatory role of prolactin on mammary epithelial cells, prolactin receptor antagonists have been found to inhibit growth of several breast tumor cell lines cultured in the absence of exogenously added human lactogens

(Fuh, et al., 1995).

Local production of prolactin in mammary epithelial cells may explain the previously noted lack of correlation between circulating prolactin levels and breast cancer risk. Furthermore, if autocrine prolactin stimulates growth of breast cancer cells, this would also explain the disappointing results with the use of dopaminergic drugs to suppress pituitary prolactin secretion.

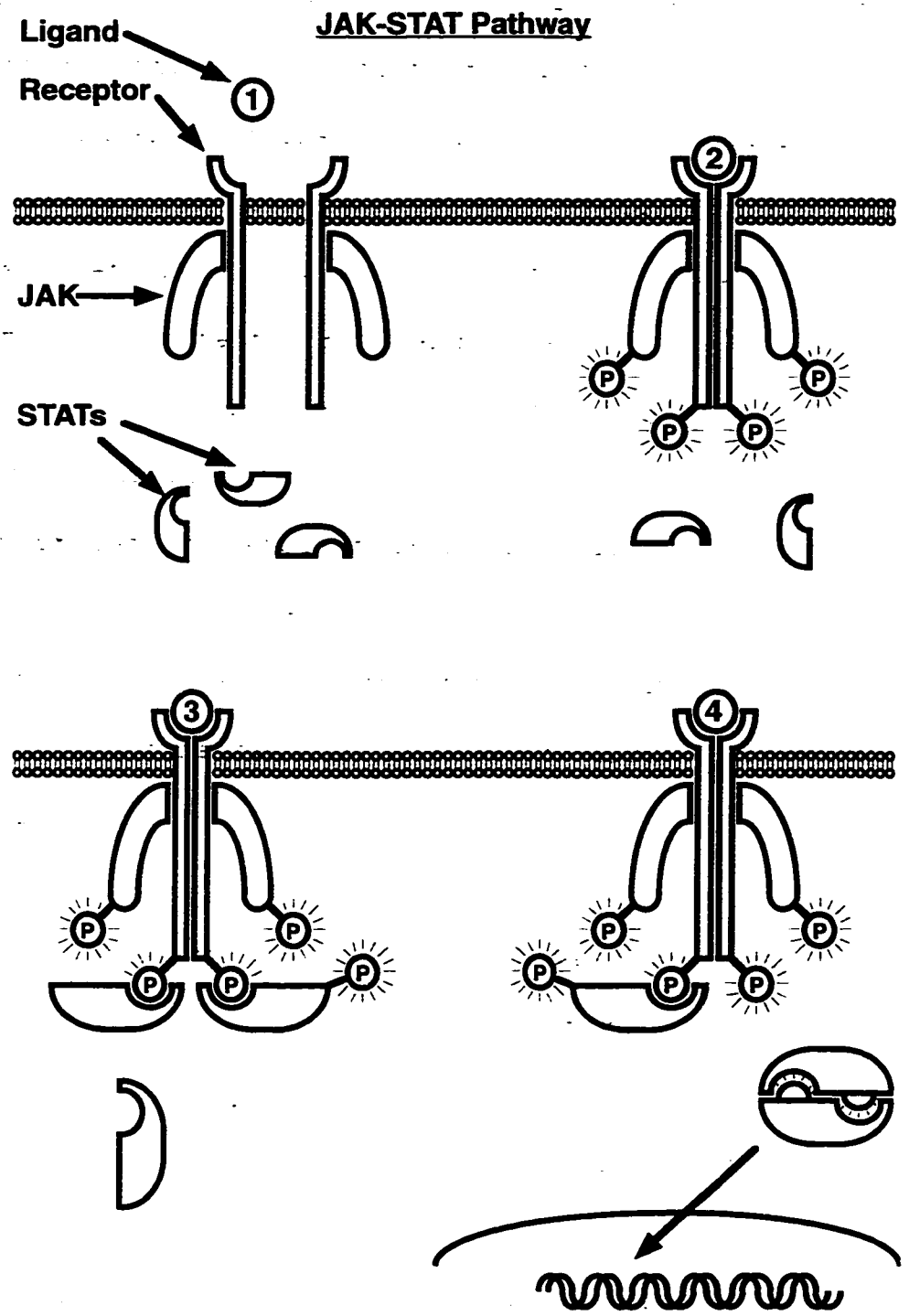
ACTIVATION OF THE JAK-STAT SIGNALING PATHWAY

To establish the extent of prolactin's and the prolactin receptor's involvement in the etiology and progression of human breast cancer, a better understanding of signal transduction and gene regulation by prolactin is needed. Significant progress has been made in this area over the past five years (fig. 2).

Prolactin: A multifunction peptide hormone

Prolactin is a 22 kilodalton peptide hormone synthesized and released by anterior pituitary lactotrophs. Control of prolactin secretion is exerted by the hypothalamus under the ultimate control of dopaminergic neurons. Prolactin regulates

Fig. 2. A generally accepted model for JAK-STAT signaling. Although some classes of cytokines may employ slight variations, the following scheme depicts the generally accepted mechanism of signal transduction used by many peptide hormones. First, binding of the ligand to its cognate receptor induces dimerization of two transmembrane receptor chains (1). Preassociated with the cytoplasmic domains of the receptors are members of the Janus kinase (JAK) family of tyrosine kinases. Upon receptor dimerization, two JAK molecules phosphorylate (P) one another as well as critical tyrosine residues on the cytoplasmic domain of the receptor (2). Members of the signal transducer and activator of transcription (STAT) family are then recruited to the activated receptor complex and themselves become phosphorylated by the JAKs (3). Two activated STAT proteins then dissociate from the receptor, dimerize and translocate to the nucleus where they activate gene transcription by binding to specific response elements in target gene promoters (4).


JAK-STAT Pathway
Ligand
Receptor
JAK
STATs
1
2
3
4
P

a variety of physiological processes including: reproduction and lactation; growth and morphogenesis; immunoregulation; metabolism; behavior; and water and salt balance. In humans, the best characterized role of prolactin, however, is its ability to induce lobuloalveolar growth in the mammary gland and to stimulate postpartum lactogenesis.

Prolactin is related to growth hormone and its placental homolog, human chorionic somatomammotropin, sharing approximately 35% amino acid sequence homology with these two proteins. Prolactin and growth hormone are believed to have arisen by gene duplication approximately 400 million years ago, while placental lactogen and growth hormone appear to be separated phylogenetically by only 10 million years, arising from intrachromosomal DNA exchange on chromosome 17 (Cooke, et al., 1981).

Molecular biology of the prolactin receptor

The receptors for prolactin belong genetically to a family of transmembrane cytokine receptors that includes growth hormone, erythropoietin, ciliary neurotrophic factor, oncostatin M, leukemia inhibitory factor, many of the interleukins, and the colony stimulating factors (Yoshimura, et al., 1990; Chiba, et al., 1992; O'Neal, et al., 1992;

Tanaka, et al., 1992; Quelle, et al., 1992; Vigon, et al., 1992; Yoshimura, et al., 1992; Colosi, et al., 1993). This receptor family shares similar structural elements in their extracellular domains, including four paired cysteine residues and a Trp-Ser-X-Trp-Ser motif (Bazan, F., 1990; Foxwell, et al., 1992; Miyajima, et al., 1992; Cosman, 1993), which are important for ligand-receptor interactions (Patthy, 1990; Kelly, et al., 1993). Greater diversity is displayed in the cytoplasmic domains, although limited homology has been demonstrated in membrane-proximal regions of the receptors (O'Neal, et al., 1992). Most importantly, none of these receptors possess any catalytic activity intrinsic to the cytoplasmic domain, in contrast to members of the EGF or insulin family (Ullrich, et al., 1990; Kazlauskas, et al., 1993; Valius, et al., 1993).

More specifically, the human prolactin receptor originally was cloned from a cDNA library obtained from the human breast cancer cell line T47D (Boutin, et al., 1988). The transcript encoded a transmembrane protein with an extracellular domain of 225 amino acids, a single transmembrane domain of 22 amino acids and a relatively large intracellular domain of 350 amino acids. The glycosylated form of this molecule has an apparent molecular weight of 88-

90 kDa. In rats and mice, short forms of prolactin receptors, that arise by alternative splicing, have also been cloned. To date three naturally occuring forms of prolactin receptors have been cloned from mammalian cells. They result from alternate splicing of mRNA, and differ only in their cytoplasmic domains (Ouhtit et al., 1993; Buck, et al., 1992). The long form of the rat prolactin receptor, which comprises 591 amino acids, is capable of transducing signals when transfected into Chinese hamster ovary cells along with a reporter gene construct. A functional role for the short form (291 amino acids) has yet to be demonstrated (Ouhtit, et al., 1993). The third form is an intermediate between the long and short form, and was cloned from rat Nb2 lymphoma cells. It is a deletion mutant of the long form of the prolactin receptor, lacking a 196 amino acid internal segment near the carboxy-terminal end of the cytoplasmic domain (Ali, et al., 1991). The Nb2 form of the prolactin receptor is fully capable of conferring the prolactin response that includes proliferation and milk protein induction.

Activation of Janus tyrosine kinases

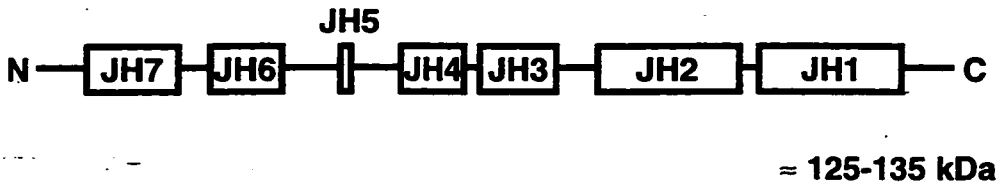
The initial molecular event causing activation of prolactin receptors is ligand-induced homodimerization

(Elberg, et al., 1990; Rui, et al., 1994). Based upon the crystal structure of its close relative, growth hormone, prolactin is believed to simultaneously bind two molecules of its cognate receptor (De Vos, et al., 1992; Ultsch, et al., 1991). Ligand-induced receptor dimerization causes aggregation of the cytoplasmic tyrosine kinase, JAK2. Like the other Janus kinases, JAK2 was found to be preassociated with the cytoplasmic domain of the prolactin receptor (Rui, et al., 1992; Rui, et al., 1994). Upon receptor dimerization, two molecules of JAK2 are brought together in close proximity, thereby inducing transphosphorylation and activating JAK2 tyrosine kinase activity (Schlessinger, et al., 1992; Stahl, et al., 1993).

Because they possess two tandem tyrosine-kinase domains (fig. 3), the JAKs were given the name Janus kinase, after the two-faced Roman god of gates and doorways. Only the most carboxyl-terminal of these kinase domains is believed to be functional; the significance of the second domain is not known. Surprisingly, the JAKs lack src homology 2 (SH2) and SH3 domains. The four members of the family, which include TYK2, JAK1, JAK2, and JAK3, range in molecular weight from 125-135 kDa. There are seven conserved JAK homology domains distributed throughout the length of the proteins giving them

Fig. 3. Homology domains of JAK family proteins. To date, there are four known members of the JAK family - TYK2, JAK1, JAK2, and JAK3 - ranging in molecular weight from 125-135 kDa. These contain seven conserved JAK homology (JH) domains giving the proteins an overall sequence identity of 35-45%. The JAKs possess two tandem tyrosine-kinase domains, although only the most carboxyl-terminal of these kinase domains is believed to be functional; the significance of the second domain is not known. The amino-terminus is important for receptor binding.

As shown below, each member of the JAK family can become activated by several peptide hormones.



Jak1	IFNs, γ-C cytokines, others
Jak2	many cytokines
Jak3	γ-C cytokines
Tyk2	IFNα, IFNβ, IL-10, IL-12, others

an overall sequence identity of 35-45%. The amino-terminus is important for receptor binding.

The initial studies of prolactin-responsive lymphoid cell lines suggested that JAK2 was the principal mediator of prolactin-induced signal transduction (Rui, et al., 1994). Based upon studies of a series of prolactin target cells, however, prolactin has been demonstrated to activate multiple JAK kinases, including JAK1, JAK2 and TYK2, in a cell-dependent manner. Evidence from mammary epithelial cell lines suggests that prolactin receptors interact in a promiscuous manner with available cellular JAK kinases (Rui, submitted). Establishing the mechanisms of how individual cytokines and hormones can activate multiple JAKs in a cell-dependent manner is essential for understanding specificity and branching of signals from receptor complexes.

STATS - Signal Transducers and Activators of Transcription

In cells at rest, the JAKs are preassociated with the cytoplasmic domains of receptor chains, but remain catalytically inactive. Upon ligand binding, the receptor-associated JAKs first phosphorylate one another, then the cytoplasmic domain of the receptor, as well (Lebrun, et al., 1994; Wakao, et al., 1994; Kirken, et al., 1993; Minami, et

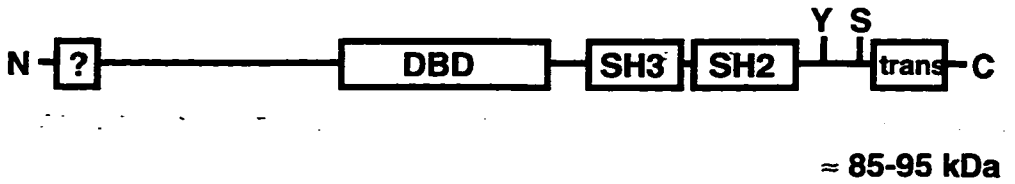
al., 1994). This invites phosphotyrosyl-binding proteins, such as members of the signal transducer and activator of transcription (STAT) family, to specifically associate with the activated receptor complex via their SH2 domains (Schlessinger, 1994; Pawson, 1995). After being recruited to the receptor complex, the STATs are themselves phosphorylated by the JAKs. Two such activated STAT proteins then dimerize and translocate to the nucleus where they activate gene transcription by binding to prolactin response elements in target gene promoters.

To date, seven members of the STAT family of transcription factors have been discovered, ranging from approximately 750 to 850 amino acids in length (fig. 4). Generally, the highest degree of homology (28-40%) is found within the first 700 amino acids, which includes a central DNA-binding domain, an SH2 domain, an SH3-like domain, a conserved tyrosine residue, and, in some cases, a conserved serine residue. The carboxyl terminus is less well-conserved but has been found to be important for transcriptional activation.

Perhaps most critical for signal specificity, the SH2 domains are conserved protein motifs that recognize and bind to particular tyrosine phosphorylated protein sequences

Fig. 4. Homology domains of STAT (Signal Transducer and Activator of Transcription) family proteins. To date, there are seven known members of the STAT family ranging in molecular weight from 85-95 kDa. Conserved domains include: a central DNA-binding domain (DBD) important in promoter recognition; an SH2 domain which recognizes specific phosphotyrosyl sequences on target receptors; an SH3-like domain whose function remains unknown; and critical tyrosine residue important for STAT activation. The carboxyl terminus contains a moderately-conserved transactivation domain (trans).

As shown below, some STATs are activated only by select peptide hormones, whereas others appear to be less specific.



STAT1	IFNs, many other cytokines and growth factors
STAT2	type I IFNs
STAT3	many cytokines
STAT4	IL-12, type I IFNs
STAT5a	many hormones, interleukins, CSFs
STAT5b	many hormones, interleukins, CSFs
STAT6	IL-4

(Songyang, et al., 1994; Pawson, T., 1995). In the case of STAT signaling, the SH2 domains of different STAT proteins recognize unique and specific phosphotyrosyl sequences on their target receptors. Marengere and colleagues have shown that the activity and specificity of these SH2 associations are dependent on the sequence directly carboxy-terminal of the phosphotyrosine residue, as well as on the sequence of the SH2 domain itself (Marengere, et al., 1994). Beyond mediating binding of the STAT to the receptor, the SH2 domain is also critical for STAT dimer formation (Ihle, et al., 1995).

Other domains of the STAT proteins are less-well characterized. Using mutational analysis, the DNA binding domain was localized to the region between amino acids 400 and 500 (Darnell, 1996). The SH3 domain is less well-conserved and its function remains unknown, although some have speculated that it may be important in stabilizing STAT dimers.

In mammary epithelial cells, the principal mediator of the prolactin response is STAT5. Originally termed mammary gland factor (MGF), STAT5 was discovered by footprinting analysis of a strongly conserved sequence element in the region between -80 and -100 of the milk protein gene, beta-

casein (Wakao, et al. 1994). Using sequence-specific DNA-affinity chromatography, STAT5 was then isolated from mammary epithelial cells of lactating rats.

More recently, STAT5 was discovered to comprise two distinct, but highly homologous isoforms, the 94 kDa STAT5a and 92 kDa STAT5b, which are encoded by separate genes (Liu, et al., 1995). Differing most in their carboxy-termini, STAT5a and STAT5b otherwise share greater than 96% sequence homology, and it has been suggested that they may have redundant or identical functions.

In contrast, STAT5a and STAT5b have only 42-43% sequence homology with their next closest relative in the STAT family, STAT6 (Liu, et al., 1995). Both STAT5a and STAT5b can be activated by many of the same cytokines, including prolactin, growth hormone, EGF and others. Indeed, no functional differences between STAT5a and STAT5b have been defined to date (Meyer, et al., 1998). Interestingly, however, tissue expression of the two proteins, while similar, does not overlap completely: STAT5a expression is high in mammary gland; STAT5b is high in muscle (Liu, et al., 1995). Further, it has recently been shown that STAT5a-deficient and STAT5b-deficient mice have different phenotypes (Liu, et al., 1997; Udy, et al., 1997).

The RAS/MAPK pathway

The RAS/MAPK pathway is another signaling pathway used by prolactin, and contains a series of oncogenic proteins whose activation ultimately stimulates transcription factors FOS and JUN (Erwin, et al., 1995; Clevenger, et al., 1994; Carey, et al., 1995). In contrast to STAT activation by prolactin, the RAS/MAPK pathway is usually linked to stimulation of cell proliferation (Bogushki, et al., 1993). The RAS-pathway includes a distinct cascade of predominantly serine/threonine kinases and mediates signals from the cell membrane to the nucleus (Moodie, et al., 1993). In breast cancer cells, prolactin activates RAS via recruitment of signaling proteins SHC, GRB2 and SOS, in a fashion similar to that observed for other growth factor receptors (Das, et al., 1996; Erwin, et al., 1995).

Cancer and the JAK-STAT pathway

Given its critical involvement in mediating cytokine regulation of growth, differentiation, and apoptosis, it is not surprising that the JAK-STAT pathway has been implicated in carcinogenesis and tumor progression. While the reports of the loss or mutation of JAKs or STATs being directly associated with human cancer are currently limited to a JAK2-

fusion protein (Peeters, et al., 1997), there is substantial evidence linking dysregulation of the JAK-STAT pathway with malignant transformation.

The first such case discovered involved the transformation of HTLV-I-infected T-cells. Activated in normal T-cells only in response to IL-2, the signal components JAK1, JAK3, STAT3 and STAT5 are constitutively activated in HTLV-I-transformed cells, allowing IL-2-independent growth (Migone, et al., 1995). Others have made similar reports involving constitutive activation of the JAK-STAT pathway in transformed B lymphocytes (Danial, et al., 1995). In fact, JAK-specific tyrosine kinase inhibitors have already found use in the clinic against acute lymphoblastic leukemia, in which constitutive JAK2 activation has been demonstrated (Meydan, et al., 1996).

There is also mounting evidence of JAK-STAT pathway dysregulation in breast cancer. It was recently demonstrated that, while overall STAT DNA-binding activity is low in normal breast and benign lesions, it is statistically elevated in breast cancer samples (Watson, et al., 1995). Indeed, constitutive activation of Stat3 was found in five of nine breast cancer cell lines tested (Garcia, et al., 1997), a finding we have also observed (Rui, et al., submitted).

ENDOCRINE- AND CYTOKINE-BASED THERAPY IN BREAST CANCER

As outlined above, mammary growth and differentiation are regulated by the complex interactions of numerous steroid hormones and cytokines, many of which are implicated in mammary tumorigenesis and progression. Although the molecular biology of many of these factors is only beginning to be understood, endocrine- and cytokine-based therapies for breast cancer have been used with varying success for decades.

Antiestrogens

Given both estrogen's critical role in mitogenesis in the normal breast, and its demonstrated effects as a tumor promoter in breast cancer, it is not surprising that antiestrogens find use as first-line agents in endocrine therapy for breast cancer. The antitumor effects of estrogen receptor antagonists, such as tamoxifen and toremifene, result from the drugs' ability to down-regulate estrogen-mediated growth factors, thus inhibiting tumor cell proliferation (Fisher, et al., 1996).

Clinically, tamoxifen has been found to benefit patients of almost all types: those with either local or advanced

disease; those who are either pre- or post-menopausal; and those who are receiving chemotherapy and not (Fisher, et al., 1996). Indeed, tamoxifen is currently under study for use in the chemoprevention of breast cancer. Recently, preliminary data from the Breast Cancer Prevention Trial (BCPT) demonstrated a 45% reduction in breast cancer incidence among women who took the drug and were considered at high-risk of developing the disease (NCI press release, March 1998).

Unfortunately, not all patients respond to antiestrogen therapy; a substantial portion of breast cancers are estrogen receptor negative, particularly in advanced stages. In fact, for reasons not yet known, even many estrogen-receptor positive tumors fail to respond to tamoxifen (Fisher, et al., 1996). Clearly, more research is needed elucidate the precise mechanisms of antiestrogen action, and its interactions with other steroid hormones and cytokines.

Glucocorticoids

Glucocorticoid hormones are frequently included in various regimens of combination chemotherapy of breast cancer, and have a beneficial effect on overall survival time (Stewart, 1982; Eastern Cooperative Oncology Group, 1984; Rubens, 1988). Similarly, a trial of radiation treatment plus

or minus prednisone found that radiation and prednisone together had a significant increase over radiation alone in disease-free interval and overall survival in premenopausal women over age 45, but not in postmenopausal women (Meakin, 1983; Ingle, 1991; Fentiman, 1994). Treatment of breast cancer with glucocorticoids alone resulted in a moderate response rate of approximately 25% (Manni, 1989), suggesting that a subpopulation of these cancers are glucocorticoid-sensitive. Little is known about the molecular mechanisms underlying the therapeutic responses of breast cancer to dexamethasone treatment (Hundertmark, 1997). However, several experimental rodent model systems have shown that glucocorticoid treatment protects against the mammary tumor promoting effect of prolactin (Chen, 1976). This suppression by glucocorticoids of prolactin-induced mammary tumor growth appears to be direct and not mediated by inhibition of pituitary prolactin secretion or by a reduction in mammary cell prolactin receptors (Aylesworth, 1980), suggesting a molecular interaction between glucocorticoids and prolactin downstream of prolactin receptor activation.

One of the specific goals of this thesis was therefore to investigate the effect of glucocorticoids on prolactin signal transduction in mammary cells, with an emphasis on

STAT5 transcription factors which are critical for breast epithelial differentiation. This is important because a better understanding of the interaction between prolactin and glucocorticoid hormones could lead to the more rational use of glucocorticoids in differentiation therapy for breast cancer.

Interferons

Cytokines of the interferon family represent a second class of biological agents used in cancer therapy. Type I interferons (interferon-alpha and interferon-beta) in particular are currently used successfully as adjuvant anticancer treatment, particularly in chronic myelogenous leukemia, malignant melanoma, low grade lymphoma, multiple myeloma, and midgut carcinoids (McLeod, et al., 1990; Einhorn, et al., 1993; Hansen, et al., 1992; Friedman, et al., 1987). It is believed that, by prolonging the cell cycle, interferons retard growth and proliferation of tumor cells (Fisher, et al., 1996). In advanced breast cancer, type I interferons have also shown therapeutic promise by enhancing the effect of tamoxifen by up-regulating estrogen receptor expression (Seymour, et al., 1993; Macheledt, et al., 1991). However, due to inconsistent clinical responses

this application has remained investigational and research efforts have focused on improving the efficacy of type I interferons in breast cancer (Einhorn, et al., 1996).

Recent progress in understanding the mechanisms of signal transduction and gene regulation underlying the antiproliferative effects of interferons has identified the critical involvement of STAT transcription factors, which translocate to the nucleus upon activation of interferon receptors (Darnell, 1997; Darnell, et al., 1996). Interestingly, the limited set of seven currently known STATs is shared by a large number of hormones and cytokines to mediate diverse biological effects (Ihle and Kerr, 1995; Schindler, et al., 1995). A certain level of signal specificity is achieved by receptor-dependent differences in selection of STAT proteins, as well as in patterns of STAT homo- and hetero-dimerization (Darnell, et al., 1994; Ihle and Witthuhn, 1995; Finbloom, et al., 1995; O'Shea, 1997). However, a potential cost of this economical sharing of STAT mediators is competition between different receptor systems for limited intracellular levels of overlapping STAT proteins (Bluyssen, et al., 1995).

Specifically, since the antiproliferative effect of type I interferons is mediated by STAT1-STAT2 heterodimers (Li, et

al., 1996; Chin, et al., 1996; Bromberg, et al., 1996), type I interferon-induced growth inhibition may be counteracted by other hormones and cytokines which also use STAT1. Since prolactin is a potent activator of STAT1, but not STAT2 (DaSilva, et al., 1996), this mammotropic hormone is a candidate type I interferon-antagonist with special relevance for the efficacy of interferon treatment of breast cancer.

A second goal of this thesis was therefore to investigate whether prolactin, a conditional mammary gland mitogen and tumor promoter, may antagonize the growth-inhibitory effect of type I interferons by competing for limited cellular levels of STAT1. This is important because it may shed light on the relative inefficiency of interferon treatment in breast cancer patients.

A third, related goal was to examine whether pretreatment of human breast cancer cells with low concentrations of the type II interferon, interferon-gamma, would enhance signal transduction by type I interferons, thus possibly enhancing their antiproliferative action. Such stimulation of type I interferon signaling by interferon-gamma has been frequently observed in cells of hematopoietic origin. This goal is important because it could lead to improved interferon-based therapy for breast cancer patients.

SPECIFIC AIMS

Aim 1

Examine whether the glucocorticoid receptor agonist, dexamethasone, may promote the terminal differentiation of human breast cancer cells by stimulating prolactin activation of the transcription factors, STAT5a and STAT5b.

Aim 2

Examine whether prolactin interferes with type I interferon signal transduction by competing for limited cytoplasmic STAT factors, thus possibly antagonizing the antiproliferative effect of type I interferons in breast cancer treatment.

Aim 3

Test if mammary tumor cell lines, like many hematopoietic cancer cells, become sensitized to the anti-proliferative effect of type I interferons by pretreatment with the type II interferon, interferon-gamma.

MATERIALS AND METHODS

Hormones and cytokines:

Ovine prolactin (NIDDK-oPRL-19, AFP-9221A) and human prolactin (NIDDK-hPRL-SIAFP-B2, AFP-2969A) were supplied by the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases (Baltimore, MD), the National Institute of Child Health and Human Development, and the U.S. Department of Agriculture. Interferons alpha, beta, and gamma were a generous gift from Dr. Andrew Larner (U.S. Food and Drug Administration). Dexamethasone, insulin and cholera toxin were purchased from Sigma (St. Louis, MO; cat. nos. D8893, I5500 and C3012, respectively). Epidermal growth factor (EGF) was obtained from Peprotech (Rocky Hill, NJ; cat. no. 100-15).

Antibodies:

Polyclonal rabbit antisera specific to peptides corresponding to the unique COOH-termini of STAT1α, STAT3, STAT5a and STAT5b were generated as described previously (Kirken, et al., 1997; DaSilva, et al., 1996). Monoclonal antiphosphotyrosine antibody 4G10 and anti-human casein were

purchased from UBI (Mountain View, CA; cat. no. 06-321) and Harlan Sera-Lab (Leicester, England; cat. no MAS 447p), respectively. Rabbit antibodies to active mitogen-activated protein kinase (MAPK) were purchased from Promega (Madison, WI; cat. no. V667A), and a mouse monoclonal anti-panERK antibody was obtained from Transduction Laboratories (Lexington, KY; cat. no. E17120/L3). Monoclonal antibodies for immunoblotting of STAT1α and STAT3 were obtained from Transduction Laboratories (Lexington, KY; cat. nos. S21120 and S21320, respectively). Horseradish peroxidase-conjugated goat antibodies to mouse or rabbit IgG were purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MD; cat. nos. 074-1806 and 074-1506, respectively).

Cell culture reagents:

Growth medium for all cell types was RPMI-1640 (Mediatech, Herndon, VA; cat. no. 15-040-LM) containing 10% fetal calf serum (Intergen, Purchase, NY; cat. no. 1020-90), 2 mM L-glutamine (Mediatech, Herndon, VA; cat. no. 25-040-LI; 50 IU/ml), penicillin-streptomycin (Mediatech, Herndon, VA; cat. no. 30-040-LI; 50 g/ml) and 5 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) buffer (Sigma, St. Louis, MO; cat. no. H-0887) pH 7.3, at

37°C with 5% CO_2. HC11 cells were cultured in complete RPMI-1640 medium supplemented with 5 µg/ml insulin and 10 ng/ml EGF. MCF-10A cells were similarly supplemented with 10 µg/ml insulin, 0.1 µg/ml cholera toxin, and 20 nM dexamethasone.

Cell lines:

Human breast cancer cell lines were obtained from American Type Culture Collection (ATCC; Rockville, MD), including: MCF-10A (cat. no. CRL-10317) transferred 1:2 weekly; MCF-7 (cat. no. HTB 22) transferred 1:2 weekly; T47D (cat. no. HTB 133) transferred 1:3 every five days; SKBr3 (cat. no. HTB 30) transferred 1:4 weekly; and BT-20 (cat. no. HTB 19) transferred 1:4 weekly. The HC11 cell line, transferred 1:5 every four days, was a gift from Dr. Gibbs Johnson (U.S. Food and Drug Administration).

Culture propagation:

Adherent mammary epithelial cells were propagated by enzymatically dissociating them from the plastic substrate with trypsin-tetrasodium ethylenediaminetetraacetate (EDTA) buffer (0.5 mg/ml porcine trypsin, 0.2 mg/ml EDTA in Hanks' Balanced Salts with phenol red; Sigma, St. Louis, MO; cat. no. T-3924). The trypsinate was inactivated with fresh growth

medium and the cells replated at the appropriate transfer ratio, as given above (Freshney, 1994).

When necessary, cells were frozen as follows: after trypsinization, cells were pelleted by centrifugation for 1 min at 1,500 g. The supernatant was removed and the cells resuspended in freezing medium containing RPMI-1640, 10% dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO; cat. no. D-5879), 10% fetal calf serum, 2 mM L-glutamine (50 IU/ml), penicillin-streptomycin (50 g/ml), and 5 mM HEPES buffer, pH 7.3. The cell suspension was aliquoted and brought slowly to -80°C (Freshney, 1994).

Labeling of probes for Northern blot analysis:

The following DNA clones were radiolabeled for use as probes for Northern blot hybridization: human c-myc, a 1.5 kb DNA fragment encoding exon II, obtained by SstI excision from clone pSP64 (Amersham, Arlington Heights, IL; cat. no. RPN-1315); human c-jun, obtained by StyI/EcoRI excision from clone pHJ (a generous gift of Drs. Robert Tjian and Dirk Bohmann, University of California at Berkeley); human c-fos, a 3.1 kb DNA fragment obtained by XhoI/NcoI excision from clone pSP65 (Amersham, Arlington Heights, IL; cat. no. RPN-1314); and human PIP, a 577 base pair DNA fragment, contained

within the EcoRI site of the plasmid pPIP-8-3 (a generous gift of Drs. R. P. Shiu and Yvonne Myal, University of Mannitoba). DNA probes were radiolabeled using a random priming kit from Stratagene (La Jolla, CA; cat. no. 300392). To a reaction mixture containing the random primers (~15 ng), deoxyribonucleotides (dATP, dTTP and dGTP at 5 mM each), and buffer, were added 40 ng of DNA and dH_2O to a final volume of 42 µl. The mixture was heated to 100°C for 5 min and pulse-centrifuged, after which 5 µl of [α-^{32}P] dCTP (6000 Ci/ml; Amersham, Arlington Heights, IL; cat. no. 10238) and 3 µl of magenta DNA polymerase (4 U/µl). Following a 10 min incubation at 37°C, the reaction was halted by adding 2 µl of 0.5 M EDTA, pH 8.0 and the probes stored at -20°C until ready for use (Kirken, et al., 1997a).

Northern blot analysis:

T47D cells were grown to confluence in growth medium, then maintained in serum-free medium with or without prolactin (10 nM), dexamethasone (1 µM) and/or insulin (5 µg/ml) for 48 h. The cells were lysed by the guanidine isothiocyanate method using Trizol solution (Ambion, Austin, TX; cat. no. 15596-018). The RNA (~10 µg) was resolved on a 1% agarose gel containing 6% formaldehyde and transferred

onto a nylon membrane (Micron Separations, Westborough, MA; cat. no. 182-002) by capillary blotting. The blots were prehybridized for 4 hr at 42°C in a solution containing 50 mM sodium phosphate, 50% formamide, saline-sodium citrate (SSC; 0.15 M NaCl, 0.015 trisodium citrate, pH 7.0; Quality Biological, Gaithersburg, MD; cat. no. 351-003-100), Denhardt's solution (0.02% each of bovine serum albumin [BSA], polyvinylpyrrolidone and Ficoll 400; Sigma, St. Louis, MO; cat. no. D-2532), 0.1% sodium dodecylsulfate (SDS; Bio-Rad, Hercules, CA; cat. no. 161-0302), and 250 µg/ml salmon sperm DNA (Pharmacia Biotech, Piscataway, NJ; cat. no. 27-4565-01). The labeled DNA probes were introduced into the prehybridization solution and allowed to hybridize for 18 h. The hybridized blots were washed 4 times with 2X SSC, 0.2% SDS, 1 mM EDTA for 5 min at room temperature, followed by two washes in 1X SSC, 0.2% SDS and 1 mM EDTA for 15 min at 50°C, and 1 wash in 0.1X SSC, 0.2% SDS and 1 mM EDTA for 30 min at 60°C (Kirken, et al., 1997a). The blots were exposed to X-Omat XAR-5 film (Eastman Kodak, Rochester, NY) with an intensifying screen for 1-5 days at -70°C. The mRNA was normalized against ethidium bromide staining of 18S rRNA by densitometric analysis using the Bio Image system (Millipore, Bedford, MA).

Induction of Casein Expression and MAPK:

Breast cancer cells were grown to confluence in growth medium, then maintained in serum-free medium with or without prolactin (10 nM), dexamethasone (1 μM) and/or insulin (5 μg/ml) for four days. The cells were harvested by scraping in ice-cold phosphate-buffered saline (PBS; Quality Biological, Gaithersburg, MD; cat. no. 114-058-100), pH 7.4, and pelleted by centrifugation at 4°C for 1 min at 2,500 g. The supernatant was removed by aspiration and the pellet immediately frozen in dry ice/methanol.

Solubilization of Proteins:

Frozen pellets from ~10^8 breast cancer cells were thawed slowly on ice, resuspended by tituration, and lysed in 1 ml of lysis buffer containing 10 mM tris-(hydroxymethyl)aminomethane(Tris)-HCl, pH 7.6 (Boehringer Mannheim, Indianapolis, IN; cat. no. 604-203), 50 mM NaCl, 5 mM EDTA (Biowhittaker, Walkersville, MD; cat. no. 17-711A), 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml aprotinin, 1 μg/ml pepstatin A and 2 μg/ml leupeptin (Sigma, St. Louis, MO; cat. nos. T-8787, P-7626, A-3428, P-4265 and L-0649, respectively). The lysates were clarified by microcentrifugation at 12,000 g for 30 min at 4°C and the

protein concentration determined by the Bradford assay (Rui, et al., 1994). In each case, 50 µg of total protein were mixed with 2x SDS sample buffer (0.125 M Tris-HCl, pH 6.8, 20% glycerol, 10% 2-mercaptoethanol, 4.6% SDS, and 0.0004% bromophenol blue) to a volume of 60 µl and separated by SDS-PAGE, as described below.

Bradford assay for protein concentration:

The protein concentration in cell lysates was determined by comparison to a BSA (Intergen, Purchase, NY; cat. no. 3220-00) standard curve (Sambrook, et al., 1989). The unknown and standard curve were prepared in a 96-well plate using 1:10 serial dilutions of 20 µl samples. Samples were diluted with 140 µl dH_2O and 40 µl of Bradford dye reagent (Bio-Rad, Hercules, CA; cat. no. 500-0006) to a total volume of 200 µl. Samples were gently mixed and incubated 5 min at room temperature. The protein concentration was determined by the relative UV absorbance at 595 nm compared to the standard curve using an automated plate-reader (Ceres 900, Bio-Tek Instruments, Winooski, VT).

Polyacrylamide gel electrophoresis:

Resolution of protein extracts was performed by

polyacrylamide gel electrophoresis (PAGE) in the presence of SDS according to the method of Laemmli (Laemmli, 1970). A 7.5% resolving gel mixture was made from 5.0 ml 4X resolving buffer (1.5 N Tris-HCl, pH 8.8, 0.4% SDS), 5.0 ml of 30% acrylamide stock (29.2% acrylamide and 0.8% bis-acrylamide; Bio-Rad, Hercules, CA; cat. no. 161-0156), 10.0 ml deionized water, 40 µl of 10% ammonium persulfate (Bio-Rad, Hercules, CA; cat. no. 161-0700) and 20 µl of N, N, N', N',-tetramethylethylene diamine (TEMED; Bio-Rad, Hercules, CA; cat. no. 161-0800). The gel was mixed and poured carefully, bubbles were eliminated by gently tapping the glass plates allowing them to rise to the top. The gel was then overlayed with 1.0 ml water-saturated sec-butanol (Aldrich, Milwaukee, WI; cat. no. B8, 591-9) and polymerized for 1 h. A stacking gel was prepared by combining 2.5 ml 4X stacking buffer (0.5 M Tris-HCl, pH 6.8, 0.4% SDS), 1.58 ml 30% acrylamide solution, 5.92 ml deionized water, 60 µl 10% ammonium persulfate and 20 µl TEMED. The sec-butanol was aspirated off and the top of the gel was washed twice with deionized water. A teflon comb was inserted in the top of the gel after addition of the stacking gel mixture, with care being taken not to create air bubbles when pouring. The stacking gel polymerized for 15 min. The teflon comb was gently removed

and 60 µl of sample loaded per well; blank wells were filled with 60 µl of 2X SDS sample buffer. Upper and lower buffer chambers were filled with electrophoresis buffer (0.025 M Tris-HCl, pH 8.3, 0.192 M glycine, 0.1% SDS). Gels were run at 24 mA constant current until the bromophenol blue dye front was at the bottom.

Gel transfer:

Protein on the gel was transferred to polyvinylidene diflouride membrane (PVDF; Immobilon, Millipore, Bedford, MA; cat. no. 1PVH 00010). Transfer was executed by equilibrating the gel in transfer buffer (0.025 M Tris-HCl, pH 8.3, 0.192 N glycine, 20% methanol) for 10 min. A sandwich was then constructed of: three layers of Whatmann 3 MM filter paper, hydrated PVDF membrane, gel, and another three layers of Whatmann 3 MM filter paper. The filter paper was presoaked in transfer buffer, while the PVDF paper was presoaked in methanol and then rinsed in transfer buffer. The gel sandwich was placed on the semi-dry transfer unit (Multiphor Novablot, Pharmacia Biotech, Piscataway, NJ). The transfer was accomplished by electrophoresis at a constant current of 195 mA for 90 min. After transfer, the blots were blocked to reduce nonspecific binding during antibody-antigen

hybridization for at least 1 h at room temperature in blocking buffer (0.02 M Tris-HCl, pH 7.6, 0.137 M NaCl, 1% BSA and 0.01% sodium azide). The blocked membranes were then ready for immunoblot analysis (Rui, et al., 1994).

Detection of casein expression:

Blots were exposed for 30 min to monoclonal anti-human casein (0.1 µg/ml) in blocking buffer. The blots were then incubated twice for 5 min in wash buffer (50 mM Tris-HCl, pH 7.6, 200 mM NaCl, 0.25% Tween 20 [polyoxyetheylenesorbitan monolaurate; Sigma, St. Louis, MO; cat. no. P-1379]), followed by incubation for 30 min with horseradish peroxidase-conjugated goat antibodies to mouse IgG at 500 ng/ml in blocking buffer, followed by four 15 min incubations in wash buffer (Rui, et al., 1994). The blots were then incubated for 1 min with enhanced chemiluminescence substrate (ECL) mixture according to the manufacturer's instructions (Amersham, Arlington Heights, IL; cat. no. RPN2106), and exposed to BioMax film for 1-5 min (Kodak, Rochester, NY; cat. no. 165 1454).

Detection of MAPK expression and activation:

Blots were exposed for 30 min to monoclonal anti-panERK

(0.1 μg/ml) or polyclonal anti-active MAPK antiserum diluted 1:2,500 in blocking buffer. The blots were then incubated twice for 5 min in wash buffer (50 mM Tris-HCl, pH 7.6, 200 mM NaCl, 0.25% Tween 20 [polyoxyetheylenesorbitan monolaurate; Sigma, St. Louis, MO; cat. no. P-1379]), then incubated for 30 min with horseradish peroxidase-conjugated goat antibodies to mouse (for anti-panERK) or rabbit (anti-active MAPK) IgG at 500 ng/ml in blocking buffer. After washing, the results were visualized by enhanced chemiluminescence and exposed to BioMax film for 1-5 min.

Preparation of affinity column:

In a 10 ml column, 2 g of cyanogen bromide (CNBr) activated Sepharose 4B resin beads (Pharmacia Biotech, Piscataway, NJ; cat. no. 17-0430-01) were equilibrated and washed with 200 ml of 1 mM HCl. The column was then washed with 200 ml of coupling buffer (0.5 M NaCl, 0.1 M $NaCO_3$, pH 8.3). STAT5a or STAT5b peptide antigen (10 mg), against which the appropriate antiserum had been raised, was dissolved in 1.5 ml of coupling buffer. Undissolved matter was removed by passing the solution through a 0.45 micron filter (Millipore, Bedford, MA; cat. no. SLHA02503). The peptide was then added to the top of the resin bed, the column sealed, and tumbled

end-over-end at room temperature for 1 h. The resin bed was then allowed to resettle for 10 min, after which excess ligand was removed by washing the column with 100 ml of coupling buffer. Any remaining active CNBr groups were blocked by slowly washing the column with 200 ml of 0.1 M Tris-HCl, pH 8.0, over a 2 h period at room temperature. In preparation for use, the column was then washed with 10 ml of buffer containing 0.5 M NaCl and 0.1 M sodium acetate, pH 4.0, followed by 10 ml of buffer containing 0.5 M NaCl and 0.1 M Tris-HCl, pH 8.0 (Harlow, et al., 1988).

Affinity purification of antisera:

Crude polyclonal rabbit antisera against STAT5a and STAT5b were purified by affinity-purification using the chromatographic column prepared above. In each case, 5 ml of the crude serum was centrifuged at 4°C for 5 min at 2,500 g then passed through a 0.45 micron filter. After being diluted 4:1 with buffer containing 0.5 M NaCl and 0.1 M Tris-HCl, pH 8.0, the filtered serum was applied to the beads. The column was sealed and tumbled end-over-end at 4°C overnight. The resin bed was then allowed to resettle for 10 min and the eluent collected. The unbound serum and all subsequent washes were saved. The beads were washed 25 ml of buffer containing

0.5 M NaCl and 0.1 M Tris-HCl, pH 8.0 to remove excess protein from the column. The purified antibody was eluted from the column with 3 bed-volumes (15 ml) of 100 mM glycine, pH 2.5 (Sigma, St. Louis, MO; cat. no. G-7403). The eluent was immediately neutralized by the dropwise addition of 1 M Tris-HCl, pH 11.0, then dialyzed against PBS with 0.02% sodium azide overnight at 4°C. The column was similarly regenerated by immediately washing with 10 ml of buffer containing 0.5 M NaCl and 0.1 M Tris-HCl, pH 8.0, followed by 10 ml of buffer containing 0.5 M NaCl and 0.1 M sodium acetate, pH 4.0. For long-term storage, the column was washed with 500 ml of 10 mM Tris-HCl, pH 7.5 supplemented with 0.01% methiolate (Harlow, et al., 1988).

Determination of antibody concentration:

Antibody concentration was determined by reading the absorbance at 280 nm using a UV spectrophotometer (DU-7, Beckmann, Fillerton, CA). For IgG antibodies, 1 absorbance unit is equivalent to an antibody concentration of approximately 0.75 mg/ml (Harlow, et al., 1988).

Dexamethasone Induction and Prolactin Stimulation:

Breast cancer cells were grown to confluence and

subsequently incubated in serum-free medium with or without dexamethasone (1 μM) for the length of time indicated (time-course experiment) or for four days (all other experiments). Cells were then stimulated for 15 min with ovine prolactin (100 μM, HC11 cells) or with human prolactin (10 nM, all other cell lines) and harvested by scraping in ice-cold PBS, pH 7.4, containing 15 mM sodium pyrophosphate, 25mM sodium fluoride and 0.1 mM sodium orthovanadate (scraping buffer). The cells were pelleted by centrifugation at 4°C for 1 min at 2,500 g. The supernatant was removed by aspiration and the pellet immediately frozen in dry ice/methanol. Frozen pellets from ~10^8 breast cancer cells were thawed slowly on ice, resuspended by tituration, and lysed in 1 ml of ice-cold lysis buffer, as described above. Cell lysates were rotated end-over-end at 4°C for 60 min, and insoluble material was pelleted at 12,000 g for 30 min at 4°C.

Immunoprecipitation of STAT5a and STAT5b:

Depending on the experiment, clarified lysates were incubated by rotating end-over-end for 3 h at 4°C with affinity purified polyclonal rabbit antisera (2 μl) against STAT5a and STAT5b. Antibodies were captured by incubation for 60 min with protein A-Sepharose beads (Pharmacia Biotech,

Piscataway, NJ; cat. no. 17-0780-01), followed by three washes in ice-cold lysis buffer. Precipitated material was eluted off the beads by addition of 75 µl of 2X SDS sample buffer followed by heating to 95°C for 5 min (Rui, et al., 1994). Samples were subjected to 7.5% SDS-PAGE and transferred to PVDF, as described above.

Immunoblot analysis of prolactin-inducible STAT5a and STAT5b:

Blots were exposed for 90 min to primary antibodies diluted in blocking buffer as follows: antiphosphotyrosine mAb 4G10 (1 µg/ml); polyclonal anti-STAT5a (1:2,500); and, polyclonal anti-STAT5b (1:2,500). The blots were then incubated twice for 5 min in wash buffer, followed by incubation for 30 min with horseradish peroxidase-conjugated goat antibodies to mouse or rabbit IgG at 500 ng/ml in blocking buffer, followed by four 15 min incubations in wash buffer (Rui, et al., 1994). The proteins were visualized by ECL, as described above.

Immunoblot stripping:

When required, blots were stripped for 30 min at 60°C in buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS and 100 mM 2-mercaptoethanol, and blocked for 2 h before reblotting.

Preparation of cytoplasmic and nuclear extracts for EMSA:

After growing to confluence, breast cancer cell lines were pretreated with dexamethasone, stimulated prolactin, and harvested by scraping, as described above. The cells were pelleted by centrifugation and immediately washed once with ice-cold PBS and once with ice-cold hypotonic buffer (10 mM HEPES pH 7.9, 10 mM KCl, 1,5 mM $MgCl_2$, 0.5 mM 1,4-dithiothreitol [Sigma, St. Louis, MO; cat. no. D-5545], 1 mM sodium vanadate, 25 mM sodium fluoride, 10 mM sodium pyrophosphate, 200 µM PMSF, 5 µg/ml aprotinin, 1 µg/ml pepstatin A and 2 µg/ml leupeptin). The washed cell pellets were then lysed in hypotonic buffer containing 0.05% Nonidet P-40 (NP-40; Sigma, St. Louis, MO; cat. no N-6507). The lysates were incubated on ice for 30 min and centrifuged at 2,500 g and 4°C for 10 min. The supernatant containing the cytoplasmic protein fraction was transferred to a clean tube and glycerol added to 20% of the final volume (Kirken, et al., 1997a). Samples were immediately frozen at -70°C.

The pellet containing the nuclear protein fraction was resuspended in one half-volume ice-cold low salt buffer (10 mM HEPES pH 7.9, 25% glycerol, 20 mM KCl, 1,5 mM $MgCl_2$, 0.5 mM 1,4-dithiothreitol, 0.2 mM EDTA, 1 mM sodium vanadate, 25 mM sodium fluoride, 10 mM sodium pyrophosphate, 200 µM PMSF, 5

μg/ml aprotinin, 1 μg/ml pepstatin A and 2 μg/ml leupeptin) to which one half-volume high salt buffer (10 mM HEPES pH 7.9, 25% glycerol, 800 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM 1,4-dithiothreitol, 0.2 mM EDTA, 1 mM sodium vanadate, 25 mM sodium fluoride, 10 mM sodium pyrophosphate, 200 μM PMSF, 5 μg/ml aprotinin, 1 μg/ml pepstatin A and 2 μg/ml leupeptin) was then added. The lysates were incubated on ice for 30 min and centrifuged at 2,500 g and 4°C for 15 min. The supernatant containing the nuclear protein fraction was transferred to a clean tube and immediately frozen at -70°C.

End-labeling of DNA probes:

Equal amounts of complementary single-stranded oligonucleotides, synthesized by Michael Flora (BIC, USUHS), were dissolved in 125 mM KCl at a concentration of 1 mg/ml. The reaction mixure was incubated at 100°C for 3 min, then 50°C for 1 h. A reaction mixture containing 250 ng of double-stranded oligonucleotide, 2 μl of T4 polynucleotide kinase (10 units/μl; New England Biolabs, Beverly, MA; cat. no. 201S), 5 μl of ^{32}P-ATP (6000 Ci/ml; Amersham, Arlington Heights, IL; cat. no. 10218) in 70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$ and 5 mM 1,4-dithiothreitol was incubated for 45 min at 37°C. After cooling to room temperature, the reaction mixture was

extracted once with phenol/chloroform/isoamyl alcohol (25:24:1 v/v; Gibco BRL, Gaithersburg, MD; cat. no. 15593-049) and purified by column chromatography. The sample was placed on a bed of Sephadex G-25 DNA grade F resin which had previously been equilibrated in accordance with the manufacturer's instructions (Pharmacia Biotech, Piscataway, NJ; cat. no. 27-5325-01). The microspin column was then centrifuged at 735 g for 2 min and the radioemission of 1 µl of the purified sample tested. The probe was then diluted to 2.5 x 10^5 cpm/µl, and stored at -20°C until ready for use.

DNA-protein binding reaction:

For the electrophoretic mobility shift assay (Wilson, et al., 1992), 1 ng of double-standed oligonucleotide corresponding to the prolactin response element of the the rat beta-casein (5' agatttctaggaattcaaatc 3') gene was end-labeled using polynucleotide kinase and [γ-^{32}P] ATP, as described above. The DNA-protein binding reactions were performed in a 30 µl mixture containing 10 µg of cytoplasmic or 5 µg of nuclear protein (as determine by the Bradford assay above) and 1 µg of double-stranded poly dI:dC (Boehringer Mannheim, Indianapolis, IN) in 12 mM HEPES (ph 7.9), 60 mM KCl, 0.5 mM 1,4-dithiothreitol, 12% glycerol and

2.5 mM $MgCl_2$. After 1 h on ice, samples were incubated with 1 µl ^{32}P-labeled beta-casein probe (15,000 cpm) and incubated for 20 min at room temperature with bromophenol blue 0.07%. The samples were then resolved by non-denaturing polyacrylamide gel electrophoresis.

EMSA analysis of protein binding to beta-casein promoter:

A 5% native resolving gel mixture was made from 0.6 ml 10X TBE (890 mM Tris-borate, pH 8.3, 890 mM boric acid, 20 mM EDTA; Quality Biological, Gaithersburg, MD; cat. no. 351-001-130) buffer, 3.7 ml of 30% acrylamide stock, 2.2 ml of 50% glycerol, 15.5 ml deionized water, 110 µl of 10% ammonium persulfate, and 22 µl of TEMED. The gel was mixed and poured carefully, bubbles were eliminated by gently tapping the glass plates allowing them to rise to the top. A teflon comb was inserted in the top of the gel with care being taken not to create air bubbles, and the gel polymerized for 1 h. The teflon comb was gently removed and the gel mounted in the electrophoresis apparatus. The upper and lower buffer chambers were filled with 0.25X TBE electrophoresis buffer, and the gel prerun in 0.25x TBE buffer at 4-10°C for 1.5 hour at 270 V. Following this equilibration, 30 µl of sample was loaded per well; blank wells were filled with 60 µl of EMSA

blank buffer (10 mM HEPES pH 7.9, 10 mM KCl, 1,5 mM $MgCl_2$, 1 mM sodium vanadate, 25 mM sodium fluoride, 10 mM sodium pyrophosphate, 0.07% bromophenol blue). After loading of samples, the gels were run at room temperature for approximately 3 hours at 250 V. Gels were dried by heating under vacuum using a slab dryer (Bio-Rad, Hercules, CA; Model 443) and exposed to X-Omat XAR-5 autoradiography film with an intensifying screen overnight at -70°C.

Supershift analysis:

For supershift analysis, before addition of ^{32}P-labeled probe, extracts were incubated with serum (1 µl normal rabbit serum or polyclonal antiserum to STAT1α, STAT3, STAT5a or STAT5b) for 30 min on ice.

Interferon-pretreatment and hormone stimulation:

Subconfluent cultures of T47D, BT20 and MCF-7 cells were incubated for 24 h in serum-free medium either with or without interferon-gamma (10 ng/ml), as indicated. Cells were then stimulated for 15 min with interferon-alpha (1000 U/ml), interferon-beta (1000 U/ml), prolactin (20 nM), or combinations of these, as indicated. The culture medium was removed, and the cells were harvested by scraping, as

described above. The cells were pelleted by centrifugation at 4°C for 1 min at 2,500 g. The supernatant was removed by aspiration and the pellet immediately frozen in dry ice/methanol.

Immunoprecipitation of interferon-stimulated STATs:

Frozen pellets from ~10^8 T47D, BT20 or MCF-7 cells were lysed and clarified, as described above. Depending on the experiment, clarified lysates were incubated rotating end-over-end for 3 h at 4°C with 2 µl of polyclonal anti-STAT1α, anti-STAT2, anti-STAT3, anti-STAT5a, or anti-STAT5b serum. Antibodies were captured by incubation for 60 min with protein A-Sepharose beads, as described above, followed by three washes in ice-cold lysis buffer. Precipitated material was eluted off the beads by addition of 75 µl of 2X SDS sample buffer followed by heating to 95°C for 5 min. Samples were subjected to 7.5% SDS-PAGE under reducing conditions, transferred to PVDF membrane, and incubated for at least 1 h at room temperature in blocking buffer before immunoblotting.

Immunoblotting for interferon-inducible STATs:

Blots were exposed for 90 min to primary antibodies diluted in blocking buffer as follows: antiphosphotyrosine

mAb 4G10 (1 µg/ml); anti-STAT1 mAb (0.1 µg/ml); anti-STAT3 mAb (0.1 µg/ml); polyclonal anti-STAT5a (1:2,500); and, polyclonal anti-STAT5b (1:2,500). The blots were then washed in wash buffer, followed by incubation for 30 min with horseradish peroxidase-conjugated goat antibodies to mouse or rabbit IgG at 500 ng/ml in blocking buffer, as described above. The results were visualized by enhanced chemiluminescence substrate and exposed to BioMax film for 1-5 min.

Preparation of cellular extracts for EMSA:

After reaching confluence in growth medium, T47D cells were cultured in serum-free medium for 24 hours prior to hormone treatment. Cells were then stimulated for 10 min with either interferon-beta (1000 U/ml) or prolactin (20 nM), or both. The culture medium was removed, and the cells were dislodged from the culture flask by scraping in ice-cold PBS, as described above. The cells were pelleted by centrifugation and immediately solubilized in EMSA lysis buffer (20 mM HEPES, pH 7.0, 10 mM KCl, 1 mM $MgCl_2$, 20% glycerol, 0.2% NP-40, 1 mM orthovanadate, 25mM NaF, 200 µM PMSF, 5 µg/ml aprotinin, 1 µg/ml pepstatin A and 2 µg/ml leupeptin). Lysates were incubated on ice for 20 min, then clarified by

centrifugation at 20,000-g for 20 min at 4°C.

DNA-protein binding reaction - interferon:

For the EMSA, 1 ng of oligonucleotide corresponding to the prolactin response elements of the rat beta-casein (5' agatttctaggaattcaaatc 3') gene, gamma-activated sequence (GAS) of the human IRF-1 (5' gatccatttccccgaaatga 3') gene, interferon-stimulated response element (ISRE) of the ISG15 (5' gatccatgcctcgggaaagggaaaccgaaactgaagcc 3') gene, or gamma-response region (GRR) of the FcγR1 (5' agcatgtttcaaggatttgagatgtatttcccagaaaag 3') gene that had been end-labeled as described above using polynucleotide kinase and [γ-^{32}P] ATP, were incubated with 10 μg of protein from cellular lysates in 30 μl of binding cocktail (50 mM Tris-Cl, pH 7.4, 25 mM $MgCl_2$, 5 mM 1,4-dithiothreitol, 50% glycerol) at room temperature for 20 minutes.

Samples were resolved by native polyacrylamide gel electrophoresis, and the results visualized, as described above. Similarly, supershift analysis was performed by preincubation of samples with 1 μl of either normal rabbit serum or antisera specific to STAT transcription factors, as indicated.

Interferon induction of MAPK:

Subconfluent cultures of T47D, BT20 and MCF-7 cells were incubated for 24 h in serum-free medium either with or without interferon-gamma (10 ng/ml), as indicated. Cells were then stimulated for 15 min with interferon-alpha (1000 U/ml), interferon-beta (1000 U/ml), prolactin (20 nM), or combinations of these, as indicated. The culture medium was removed, and the cells were harvested by scraping, as described above. The cells were pelleted by centrifugation at 4°C for 1 min at 2,500 g. The supernatant was removed by aspiration and the pellet immediately frozen in dry ice/methanol. Frozen pellets from ~10^8 T47D, BT20 or MCF-7 cells were lysed and clarified, as described above.The whole-cell lysates were subjected to 7.5% SDS-PAGE under reducing conditions, transferred to PVDF membrane, and incubated for 1 h at room temperature in blocking buffer before immunoblotting. Blots were exposed for 30 min to monoclonal anti-panERK (0.1 µg/ml) diluted in blocking buffer. As described above, the blots were washed and then incubated for 30 min with horseradish peroxidase-conjugated goat antibodies to mouse IgG at 500 ng/ml in blocking buffer. After washing, the results were visualized by enhanced chemiluminescence and exposed to BioMax film for 1-5 min.

MTT Assay:

T47D cells were dispensed into 96-well microtiter plates at 10^4 cells/100 µl and incubated for 24 h in growth medium. The cells were then cultured in serum-free medium either with or without interferon-gamma (10 ng/ml) for 24 h. The samples were incubated an additional 48 h in the presence of either interferon-beta (1000 U/ml), prolactin (10 nM) or both. Viable cells were measured metabolically using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method, in accordance with the manufacturer's instructions (Promega, Madison, WI; cat. no. G5421). The MTT assay measures mitochondria respiration, which for this study correlated with DNA synthesis. Fifteen microliters of the MTT dye reagent was added to each well and after an additional 4 h incubation at 37°C, 100 µl of solubilization mix was added. Colorimetric analysis was performed using a semiautomatic plate reader from Dynatech Laboratories (Chantilly, VA; MR600) by reading the relative absorbance of the blue reduction product of MTT, formazan, at 570 nm with a reference wavelength of 620 nm.

Thymidine incorporation assay:

In parallel experiments, T47D cells were dispensed into

96-well microtiter plates at 10^4 cells/100 µl and incubated for 24 h in growth medium at 37°C. The cells were then cultured in serum-free medium either with or without interferon-gamma (10 ng/ml) for an additional 24 h. Ten percent fetal calf serum was added back to the medium, and the cells stimulated with interferon-beta (1000 U/ml), prolactin (10 nM), or both, as indicated. After 24 h, [^{3}H]-thymidine (ICN; Costa Mesa, CA; cat. no. 24039.2) was added to a final concentration of 10 µCi/ml and the cells incubated 4 h at 37°C. The medium was removed and the samples washed twice with serum-free medium to remove unincorporated [^{3}H]-thymidine label (Sambrook, et al., 1989). The cells were dissociated from the substrate by 15 min incubation at 37°C with 100 µl trypsin/EDTA solution and harvested onto glass fiber filters (Millipore, Bedford, MA; cat. no. STHA096NS). The cells were washed with 500 µl of ice-cold PBS then allowed to airdry overnight. The filters were transferred using a punch press to individual scintillation vials containing 3 ml of Ready-Solv scintillation fluid (Beckmann, Fullerton, CA; cat. no. 158726). After allowing the samples to equilibrate for 1 h, the cells were analyzed for [^{3}H]-thymidine incorporation using an automated scintillation counter (LS6000TA, Beckman, Fullerton, CA).

Statistical Analysis:

For comparisons of the mean responses of several groups, one-way analysis of variance followed by Sheffe's multiple range test was employed using SPSS 6.1 (Norusis/SPSS, Inc.).

GLUCOCORTICOID MODULATION OF PROLACTIN SIGNALS

Introduction

Prolactin has been shown unequivocally to be a tumor promoter of the mammary gland by a variety of experimental approaches in rodents, including prolactin over-expressing transgenic mice (Welsh, et al., 1977; Tejwani, et al., 1991; Rana, et al., 1995; Wennbo, et al., 1997). The role of prolactin in the etiology and progression of breast cancer in humans has been controversial mainly due to the lack of a simple correlation between circulating prolactin levels and breast cancer incidence (Ingram, et al., 1991; Love, et al., 1991; Maddox, et al., 1992; Wang, et al., 1992; Zumoff, 1994; Nandi, et al., 1995), and that pharmacological suppression of pituitary prolactin secretion has had inconsistent impact on tumor growth (Peyrat, et al., 1984; Bonneterre, et al., 1990). However, several laboratories have detected local production of prolactin in rodent and human mammary epithelium and human breast carcinomas, and accumulating evidence suggests that prolactin can act as an autocrine mammary growth factor (Fields, et al., 1993; Clevenger, et al., 1995; Ginsburg, et al., 1995; Mershon, et al., 1995;

Wennbo, et al., 1997). Consistent with a local growth stimulatory role of prolactin on mammary epithelial cells, prolactin receptor antagonists inhibited the growth of several human breast tumor cell lines cultured in the absence of exogenous lactogenic hormones (Fuh, et al., 1995). Furthermore, 40-70% of human breast tumor biopsies are positive for prolactin-receptors (Bonneterre, et al., 1990; Murphy, et al., 1984), and many tumor-derived cell lines express increased levels of prolactin receptors and can proliferate in response to prolactin in vitro (Shiu, 1979; Shiu, et al., 1985; Manni, et al., 1986; Malarkey, et al., 1983; Biswas, et al., 1987). However, in the appropriate hormonal milieu, particularly in the presence of glucocorticoid hormones, prolactin becomes a differentiation factor for mammary cells (Juergens, et al., 1965; Borellini, et al., 1989; Merlo, et al., 1996). It is therefore possible that the beneficial effects of glucocorticoids in a subpopulation of breast cancer patients is due to a modulation of prolactin receptor signals.

Prolactin activates the RAS-mitogen-activated protein kinase (MAPK) pathway (Erwin, et al., 1995; Das, et al., 1996a) and the JAK2-STAT5 pathway (Rui, et al., 1994; Gouilleux, et al., 1994; Watson, et al., 1996). Whereas the

mitogenic effects of prolactin in mammary cells have been attributed to the RAS-MAPK signaling pathway, (Das, et al., 1996b; Carey, et al., 1995; Buckley, et al., 1994), prolactin-induced terminal differentiation of mammary epithelium and milk protein expression (e.g., beta-casein, beta-lactoglobulin and prolactin-inducible protein [PIP]) appear to be mediated by activation of STAT5 transcription factors (Han, et al., 1997; Groner, et al., 1995; Wartmann, et al., 1996), particularly STAT5a (Liu, et al., 1997).

Recent studies in reconstituted COS-7 cells have suggested that glucocorticoids facilitate prolactin signaling via STAT5 directly at the level of the beta-casein gene promoter, and accumulating evidence supports the concept that the glucocorticoid receptor is a ligand-activated coactivator of STAT5 transcription factors (Stocklin, et al., 1996; Schmitt-Ney, et al., 1991; Stoecklin, et al., 1997; Lechner, et al., 1997). Despite this direct interaction between the glucocorticoid receptor and STAT5 molecules observed in COS-7 cells, several days of pretreatment with glucocorticoid hormones are needed to detect an enhancement of prolactin-induced differentiation markers in mammary cells (Doppler, et al., 1990), suggesting the existence of additional levels of cooperation between glucocorticoids and prolactin.

We now report that dexamethasone can upregulate prolactin signaling via STAT5a in certain mammary cell lines. Pretreatment of the human breast cancer cell line T47D for 2-4 days led to marked enhancement of prolactin activation of the transcription factor STAT5a. Thus, dexamethasone induced a qualitative change in prolactin signals from exclusive STAT5b activation to combined recruitment of STAT5a and STAT5b, with extensive heterodimerization of the two transcription factors. This dexamethasone-dependent change in prolactin signals was associated with prolactin stimulation of terminal differentiation markers in T47D cells. Interestingly, prolactin activation of MAPK and growth-related genes c-fos, c-jun and c-myc was not affected by dexamethasone. A similar, but less marked, stimulation by dexamethasone pretreatment of prolactin-activated STAT5a was seen in MCF-7 cells. On the other hand, the two near-normal mammary cell lines, HC11 and MCF-10A, expressed equal levels of STAT5a and STAT5b in a dexamethasone-independent manner. Furthermore, STAT5a expression was lost and could not be induced by dexamethasone in the undifferentiated, estrogen receptor (ER)-negative BT-20 and SKBr3 cell lines.

These studies identify STAT5a as a target for dexamethasone regulation of potential importance for

differentiation therapy of certain mammary cancers, and may help explain the variable response rate of breast cancer patients to glucocorticoid treatment.

RESULTS

Dexamethasone critical for prolactin-induced differentiation

Dexamethasone stimulates a differentiation response to prolactin in human T47D breast cancer cells. It is well established that the hormones prolactin, hydrocortisone and insulin cooperate in the regulation of milk protein synthesis and differentiation of mammary explants and normal mammary epithelial lines (Topper, et al., 1980; Merlo, et al., 1996). However, little is known about the precise mechanism underlying the differentiating effect of glucocorticoids in human mammary cancer cells (Archer, et al., 1994; Hundertmark, et al., 1997). We first examined the ability of glucocorticoids to influence prolactin-induced differentiation of the well-characterized human mammary cancer cell line, T47D. Originally derived from a patient with a moderately differentiated infiltrating ductal carcinoma, T47D cells express receptors for estrogen, progesterone, glucocorticoids, and prolactin (Engel, et al., 1978). Insulin was also

included in these initial experiments due to its reported promotion of mammary differentiation.

When confluent monolayers of T47D cells were preincubated under serum-free conditions in the presence or absence of dexamethasone (1 μM) and/or insulin (5 μg/ml) for 48 h before addition of prolactin for 12 h, a marked increase in expression of the milk protein, casein, was detected by immunoblotting in response to prolactin in the dexamethasone-pretreated cells (fig. 5). Insulin pretreatment alone had no effect on prolactin-induced casein induction, and coadministration of insulin did not modulate the positive effect of dexamethasone (fig. 5, lanes f and h). A similar pattern was observed of dexamethasone-dependent and insulin-independent stimulation of prolactin-induced mRNA levels of another secretory protein, gross cystic disease fluid protein-15 (fig. 6, panel 1), also known as prolactin-inducible protein (PIP). Originally characterized as a prolactin-responsive gene in a variety of exocrine glands (Shiu, 1987; Myal, et al., 1991), PIP expression has also been shown to correlate positively with mammary tumor differentiation (Miller, et al., 1988; Labrie, et al., 1990). In contrast to the positive effect of dexamethasone on prolactin-induced differentiation markers, dexamethasone pretreatment did not modulate

Fig. 5. Immunoblot analysis. Dexamethasone (DEX) stimulates a differentiation response to prolactin (PRL) in human T47D breast cancer cells. Confluent T47D cells were maintained in serum-free medium with or without PRL (10 nM), DEX (1 μM) and/or insulin (5 μg/ml) for four days. Whole-cell lysates were separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with anti-human casein mAbs. Results were visualized by enhanced chemiluminescence. Note that a marked increase in expression of the milk protein, casein, was detected by immunoblotting in response to prolactin in the dexamethasone-pretreated cells. Insulin pretreatment alone had no effect on prolactin-induced casein induction, nor did coadministration of insulin modulate the positive effect effect of dexamethasone.

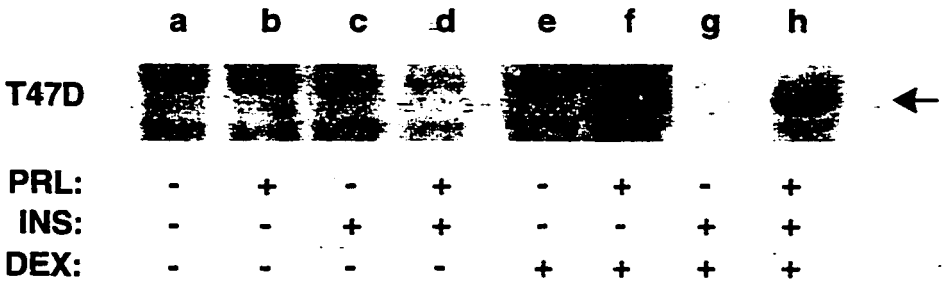
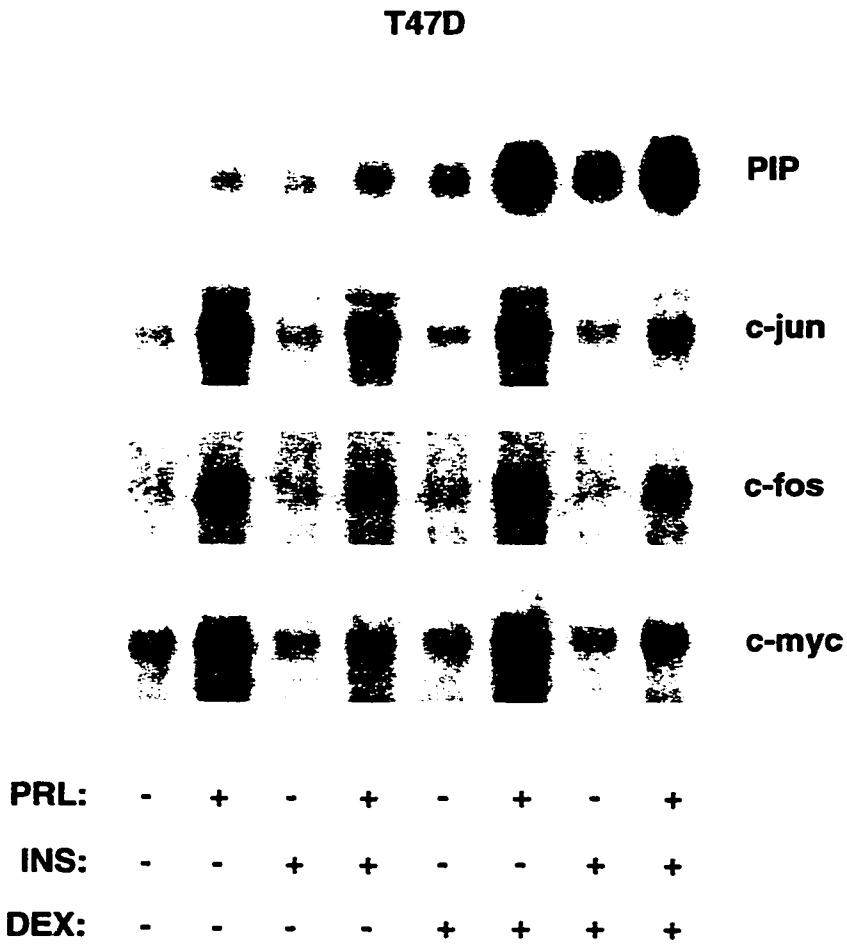

a b c d e f g h
T47D
PRL: - + - + - + - +
INS: - - + + - - + +
DEX: - - - - + + + +

Fig. 6. Northern blot analysis. Confluent T47D cells were maintained in serum-free medium with or without prolactin (PRL; 10 nM), dexamethasone (DEX; 1 μM) and/or insulin (INS; 5 μg/ml) for four days. Cells were lysed by the guanidine isothiocyanate method, the RNA (~10 μg) resolved on a 1% agarose gel, and transferred to a nylon membrane. Following 4 h prehybridization, radiolabelled DNA probes corresponding to the genes indicated were introduced and allowed to hybridize for 18 h. The blots were washed and exposed to X-ray film with an intensifying screen for 1-5 days at -70°C. The mRNA was normalized against ethidium bromide staining of 18S rRNA by densitometric analysis. Note that, while dexamethasone-dependent stimulation of the secretory protein, prolactin-inducible protein (PIP), was observed, dexamethasone pretreatment did not modulate prolactin-induction of mRNA levels for the growth-related genes c-fos, c-jun and c-myc.

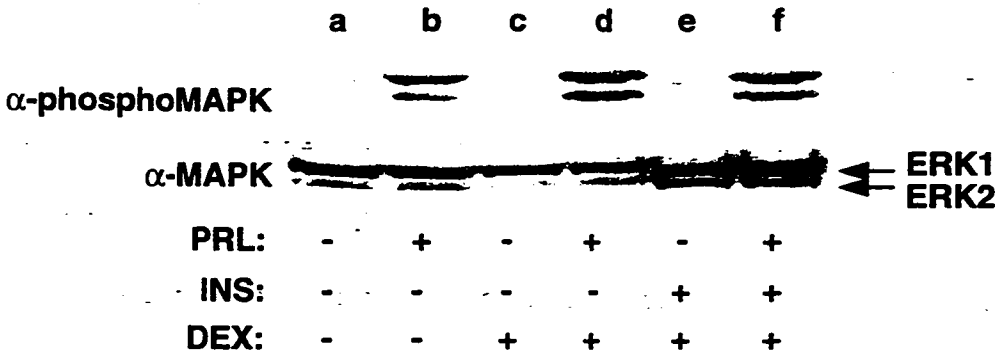

T47D
PIP
c-jun
c-fos
c-myc
PRL: - + - + - + - +
INS: - - + + - - + +
DEX: - - - - + + + +

prolactin-induction of mRNA levels for the growth-related genes c-fos, c-jun and c-myc (fig. 6, panels 2-4). On the other hand, insulin pretreatment alone or in combination with dexamethasone appeared to reduce the prolactin-induced mRNA levels of these growth-related transcription factors (fig. 6, panels 2-4, lanes c and h).

Since prolactin has been specifically suggested to stimulate growth of T47D cells via the RAS/MAPK pathway (Das, et al., 1996b; Buckley, et al., 1994), we also examined the effect of dexamethasone pretreatment on prolactin-activation of MAPK. As was the case for prolactin-induced proto-oncogene expression, 48 h of pretreatment with dexamethasone under serum-free conditions did not significantly affect prolactin-induced MAPK activation or the expression of the ERK1 and ERK2 serine kinases (fig. 7). Pretreatment with insulin and dexamethasone also did not influence prolactin-activation of MAPK, although this combination appeared to moderately elevate the expression levels of ERK1 and ERK2.

From this initial set of data we concluded that pretreatment of the human breast cancer cell line T47D with dexamethasone had a marked stimulatory effect on prolactin induction of the differentiation markers casein and PIP, but did not modulate putative growth signals via MAPK or

Fig. 7. Immunoblot analysis. Confluent T47D cells were maintained in serum-free medium with or without prolactin (PRL; 10 nM), dexamethasone (DEX; 1 μM) and/or insulin (INS; 5 μg/ml) for four days. Whole-cell lysates were separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with monoclonal anti-panERK or polyclonal anti-active MAPK antiserum. Results were visualized by enhanced chemiluminescence. Note that pretreatment with dexamethasone under serum-free conditions did not significantly affect prolactin-induced MAPK activation or the expression of the ERK1 and ERK2 serine kinases. Pretreatment with insulin and dexamethasone also did not influence prolactin-activation of MAPK, although this combination appeared to moderately elevate the expression levels of ERK1 and ERK2.


a b c d e f
α-phosphoMAPK
α-MAPK
ERK1
ERK2
PRL: - + - + - +
INS: - - - - + +
DEX: - - + + + +

transcription factors c-jun, c-fos or c-myc. Insulin appeared to be of lesser importance for prolactin induction of differentiation markers than what has been previously reported for normal mammary cells (Bolander, et al., 1981). Since STAT5 transcription factors are putative mediators of prolactin-induced differentiation in the mammary gland (Groner, et al., 1995; Wartmann, et al., 1996; Han, et al., 1997; Liu, et al., 1997), we specifically examined whether dexamethasone modulated prolactin activation of the STAT5a and STAT5b transcription factors.

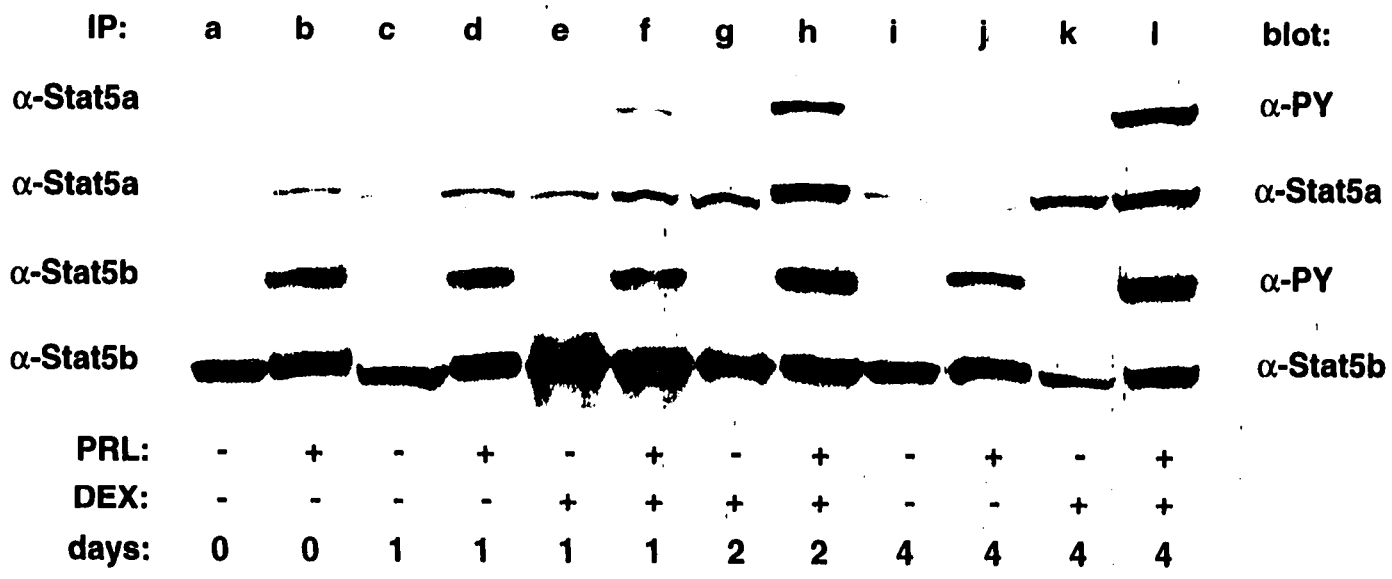
Dexamethasone upregulates activation of STAT5a in T47D cells

Pretreatment of T47D cells with dexamethasone upregulates prolactin-induced activation of STAT5a and leads to formation of STAT5a-STAT5b heterodimers. We have previously described prolactin-induced activation of STAT5 in T47D cells (Dasilva, et al., 1996). However, more detailed analysis revealed that this was a selective STAT5b activation with little or no STAT5a activation (Schaber, et al., 1998), suggesting the possible loss of a mediator of differentiation in T47D cells. This is in contrast to other prolactin target cells thus far examined, in which prolactin activates STAT5a and STAT5b equally well, including lymphoid Nb2 cells, HC11

mammary epithelial cells, prolactin receptor expressing 32D myeloid cells and Ba/F3 lymphoid cells (Merlo, et al., 1996; Kirken, et al., 1997a). Although both STAT5a and STAT5b transcription factors are able to mediate prolactin-induced beta-casein transcription when expressed individually in COS-7 cells, mammary development and differentiation is significantly more impaired in STAT5a knockout mice than in STAT5b deficient mice which are able to lactate (Liu, et al., 1997; Udy, et al., 1997).

We first examined the effect of dexamethasone pretreatment on the extent of prolactin-induced tyrosine phosphorylation of STAT5a and STAT5b and their expression levels. Preliminary experiments showed that a preincubation period of between 48 and 96 hours was required for dexamethasone to facilitate prolactin stimulation of differentiation markers in T47D cells (data not shown). Confluent T47D cells were therefore treated with or without dexamethasone for varying times up to 4 days in the absence of serum, and were then stimulated with prolactin for 15 min before analysis by immunoprecipitation and immunoblotting (fig. 8). As previously noted, in T47D cells that had not been pretreated with dexamethasone (panels 1 and 3; lanes a and b), prolactin selectively activated STAT5b with little or

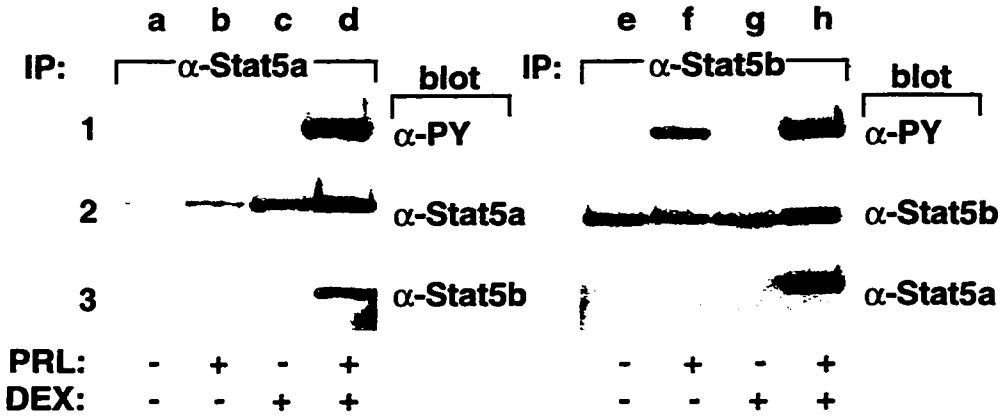
Fig. 8. Time-course experiment. Dexamethasone upregulates activation of STAT5a in T47D cells. Confluent T47D cells were incubated in serum-free medium with or without dexamethasone (DEX; 1 μM) for the length of time indicated, then stimulated for 15 min with prolactin (PRL; 10 nM). Lysates were immunoprecipitated (IP) with anti-STAT5a (α-STAT5a) or anti-STAT5b antiserum, as indicated, and separated by SDS-PAGE. Following transfer to PVDF membrane, samples were immunoblotted with anti-phosphotyrosine (α-PY), anti-STAT5a or anti-STAT5b antiserum, as indicated. Results were visualized by enhanced chemiluminescence. Note that 96 h preincubation with dexamethasone had a marked stimulatory effect on prolactin-induced STAT5a tyrosine phosphorylation (panel 1). The basal expression of STAT5a was also significantly elevated from very low levels (panel 2) to amounts approximately equal to the those of STAT5b (panel 4). There was no significant induction of STAT5b expression or tyrosine phosphorylation by dexamethasone treatment.


IP: a b c d e f g h i j k l blot:
α-Stat5a
α-PY
α-Stat5a
α-Stat5a
α-Stat5b
α-PY
α-Stat5b
α-Stat5b
PRL: - + - + - + - + - + - +
DEX: - - - - + + + + - - + +
days: 0 0 1 1 1 1 2 2 4 4 4 4

no activation of STAT5a. However, over 4 days of pretreatment, dexamethasone had a marked stimulatory effect on prolactin-induced STAT5a tyrosine phosphorylation (panel 1). The basal expression of STAT5a was also significantly elevated from very low levels (panel 2) to amounts approximately equal to those of STAT5b (panel 4). There was no significant induction of STAT5b expression or tyrosine phosphorylation by dexamethasone treatment, leading to prolactin-induced formation of significant amounts of STAT5a-STAT5b heterodimers, as visualized by reciprocal coimmunoprecipitation of STAT5a and STAT5b from cells treated with dexamethasone followed by prolactin (fig. 9, panel 3).

Although it remains to be established whether this qualitative shift in the pattern of STAT5 isoform activation alters the gene expression control by prolactin receptors, the observation that mammary gland differentiation is more affected in STAT5a-deficient mice than in STAT5b-deficient mice (Liu, et al., 1997; Udy, et al., 1997) has suggested that these two homologous proteins are functionally different. The observed induction by dexamethasone of STAT5a activation by prolactin in T47D cells thus indicated an unrecognized form of cooperation between glucocorticoid and prolactin at the level of STAT5a expression and recruitment.

Fig. 9. Immunoblot analysis. Confluent T47D cells were maintained in serum-free medium for four days with or without dexamethasone (DEX; 1 μM), then stimulated for 15 min with prolactin (PRL; 10 nM). Lysates were immunoprecipitated (IP) with anti-STAT5a (α-STAT5a) or anti-STAT5b antiserum, as indicated, and separated by SDS-PAGE. Following transfer to PVDF membrane, samples were immunoblotted with anti-phosphotyrosine (α-PY), anti-STAT5a or anti-STAT5b antiserum, as indicated. Results were visualized by enhanced chemiluminescence. Note that there was no significant induction of STAT5b expression or tyrosine phosphorylation by dexamethasone treatment, leading to prolactin-induced formation of STAT5a-STAT5b heterodimers, as visualized by reciprocal coimmunoprecipitation of STAT5a and STAT5b (panel 3).

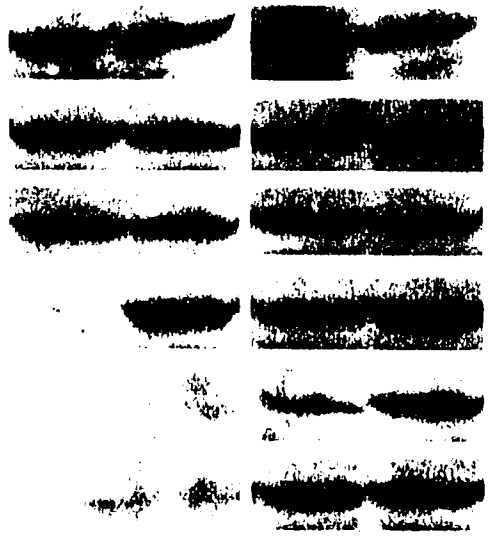

a b c d
IP: α-Stat5a
blot
1 α-PY
2 α-Stat5a
3 α-Stat5b
PRL: - + - +
DEX: - - + +
e f g h
IP: α-Stat5b
blot
α-PY
α-Stat5b
α-Stat5a
- + - +
- - + +

Cell-dependent modulation of STAT5a expression

The extent of dexamethasone modulation of STAT5a expression is mammary cell line-dependent. To determine the extent of the dexamethasone-inducibility of STAT5a expression in cell lines other than T47D, we expanded the analysis to a total of six mammary cell lines. These cells included two near-normal, spontaneously immortalized cell lines, HC11 and MCF-10A; two moderately-differentiated and ER-positive cell lines, MCF-7 and T47D; and two poorly-differentiated and ER-negative cell lines, SKBr3 and BT-20. All of these cell lines are human, except the murine HC11 line.

For these experiments, cells were grown to confluency and treated with or without dexamethasone for 4 days in serum-free medium. After harvesting, cells were lysed and 50 µg of total cell protein separated by SDS-PAGE. Immunoblotting revealed that, whereas STAT5b was expressed at comparable levels in all six cell lines examined, STAT5a expression varied markedly (fig. 10). The near-normal cell lines HC11 and MCF-10A, as well as MCF-7 cells, expressed significant levels of STAT5a independent of dexamethasone treatment. As observed earlier, dexamethasone markedly stimulated expression of STAT5a in T47D cells, but did not detectably upregulate STAT5a in the ER-negative, less-

Fig. 10. Immunoblot analysis. The extent of dexamethasone modulation of STAT5a expression is mammary cell line-dependent. Confluent breast cancer cells were maintained in serum-free medium for four days with or without dexamethasone (DEX; 1 μM). Cells were lysed and 50 μg of total cell protein separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with either anti-STAT5a (α-STAT5a) or anti-STAT5b antiserum. Results were visualized by enhanced chemiluminescence. Note that whereas STAT5b was expressed at comparable levels in all six cell lines examined, STAT5a expression varied markedly: the near-normal cell lines HC11 and MCF-10A, as well as MCF-7 cells, expressed significant levels of STAT5a independent of dexamethasone treatment. Dexamethasone markedly stimulated expression of STAT5a in T47D cells, but did not detectably upregulate STAT5a in the ER-negative, less-differentiated SKBr3 or BT-20 cells.



	ER status	tumor-igenicity	pathology
MCF-10	+	-	near-normal, human
HC11	+	-	near-normal, mouse
MCF-7	+	+	moderately differentiated
T47D	+	+	moderately differentiated
SKBr3	-	+	highly anaplastic
BT-20	-	+	poorly differentiated

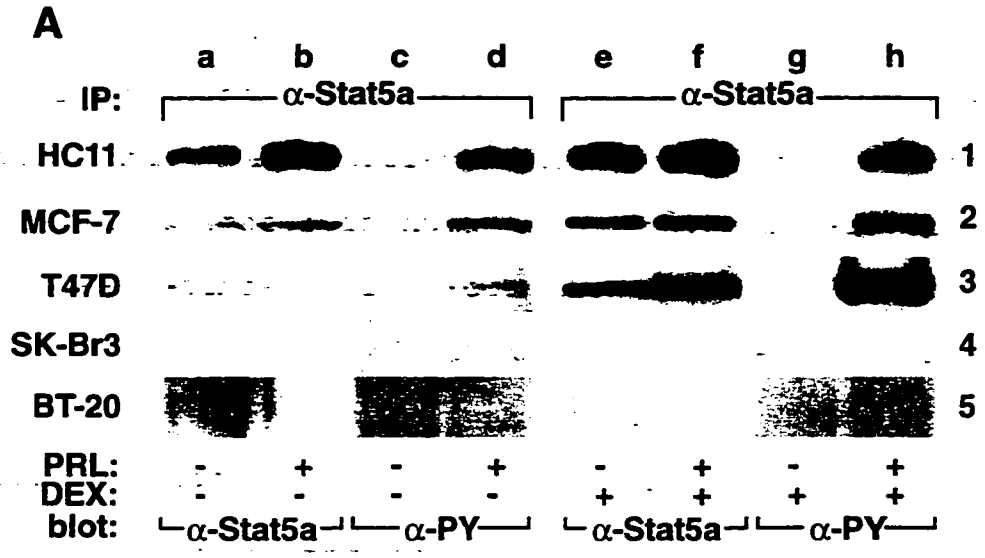
differentiated SKBr3 or BT-20 cells. The fact that SKBr3 and BT20 did not express STAT5a is of possible importance for their undifferentiated phenotype.

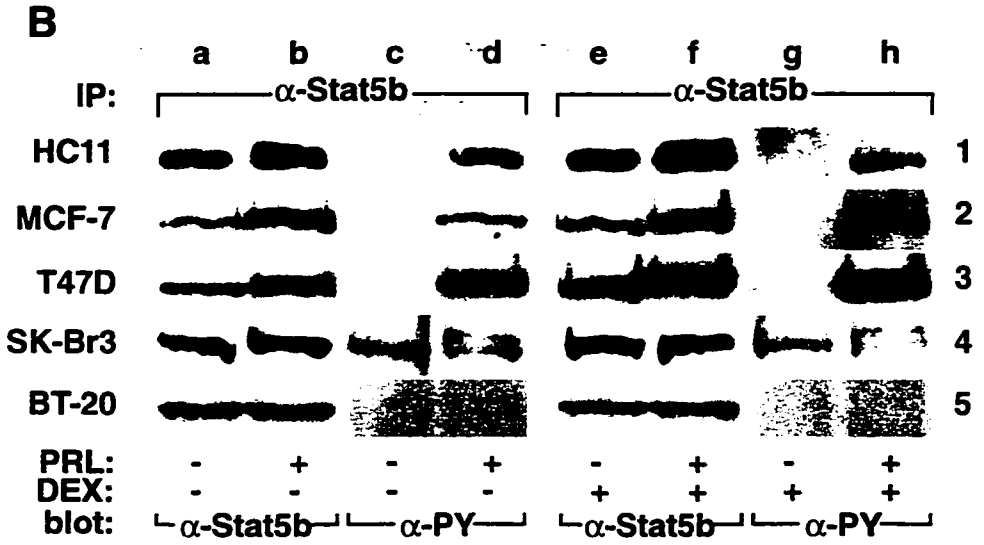
We next examined the effect of dexamethasone pretreatment on prolactin-induced tyrosine phosphorylation of STAT5a and STAT5b proteins in the six cell lines.

Effect of dexamethasone on STAT5a phosphorylation

The synergistic effect of dexamethasone on prolactin-induced STAT5a tyrosine phosphorylation is mammary cell line-dependent. Antiphosphotyrosine immunoblotting showed cell-dependent differences which corresponded well with the differences in STAT5a and STAT5b expression in response to dexamethasone pretreatment. Specifically, prolactin-inducible STAT5a tyrosine phosphorylation varied markedly between cell lines without dexamethasone pretreatment (fig. 11A, lanes a-d) and with dexamethasone pretreatment (lanes e-h). The STAT5a signal enhancement by dexamethasone pretreatment was evident in T47D cells and to a lesser extent in MCF-7 cells, the two moderately-differentiated cell lines (panels 2 and 3). No effect of dexamethasone pretreatment was seen on prolactin-induced tyrosine phosphorylation of STAT5a or STAT5b in the poorly-differentiated and ER-negative cell

Fig. 11. Immunoblot analysis. The synergistic effect of dexamethasone on prolactin-induced STAT5a tyrosine phosphorylation is mammary cell line-dependent. Confluent breast cancer cells were maintained in serum-free medium for four days with or without dexamethasone (DEX; 1 μM), then stimulated for 15 min with prolactin (PRL; 10 nM). Lysates were immunoprecipitated with anti-STAT5a (α-STAT5a) or anti-STAT5b antiserum, as indicated, and separated by SDS-PAGE. Following transfer to PVDF membrane, samples were immunoblotted with anti-phosphotyrosine (α-PY), anti-STAT5a or anti-STAT5b antiserum, as indicated. Results were visualized by enhanced chemiluminescence. Note that prolactin-inducible STAT5a tyrosine phosphorylation varied markedly between cell lines without dexamethasone pretreatment (lanes a-d) and with dexamethasone pretreatment (lanes e-h).


A
a b c d e f g h
IP: α-Stat5a α-Stat5a
HC11 1
MCF-7 2
T47D 3
SK-Br3 4
BT-20 5
PRL: - + - + - + - +
DEX: - - - - + + + +
blot: α-Stat5a α-PY α-Stat5a α-PY


B
a b c d e f g h
IP: α-Stat5b α-Stat5b
HC11 1
MCF-7 2
T47D 3
SK-Br3 4
BT-20 5
PRL: - + - + - + - +
DEX: - - - - + + + +
blot: α-Stat5b α-PY α-Stat5b α-PY

lines, SKBr3 and BT-20. The near-normal HC11 cells showed marked prolactin-induced tyrosine phosphorylation of STAT5a and STAT5b independent of dexamethasone pretreatment, whereas no prolactin induced STAT5 signal was seen in the MCF-10A cell line (data not shown), possibly due to lower prolactin receptor expression.

Parallel examination of STAT5b activation (fig. 11B) showed prolactin-inducible STAT5b tyrosine phosphorylation responses in each of the cell lines, with exception of BT-20, which has a very weak prolactin response despite its high prolactin receptor expression (Shiu, 1979; Rui, et al., 1998; Schaber, et al., 1998). As seen in fig. 10, STAT5b was expressed at comparable levels in each of the cell lines and dexamethasone pretreatment only moderately enhanced the extent of prolactin-induced tyrosine phosphorylation of STAT5b in T47D and MCF-7 cells (fig. 11B, panels 2 and 3). STAT5b undergoes a discernible gel-retardation upon tyrosine and serine phosphorylation (Kirken, et al., 1997a, b), and the dexamethasone-enhanced tyrosine phosphorylation was reflected in an increased ratio of the shifted over the unshifted STAT5b bands (eg., panel 2, lanes b and f). In SKBr3 cells, prolactin induced only very moderate tyrosine phosphorylation of STAT5b as compared to the other cells.

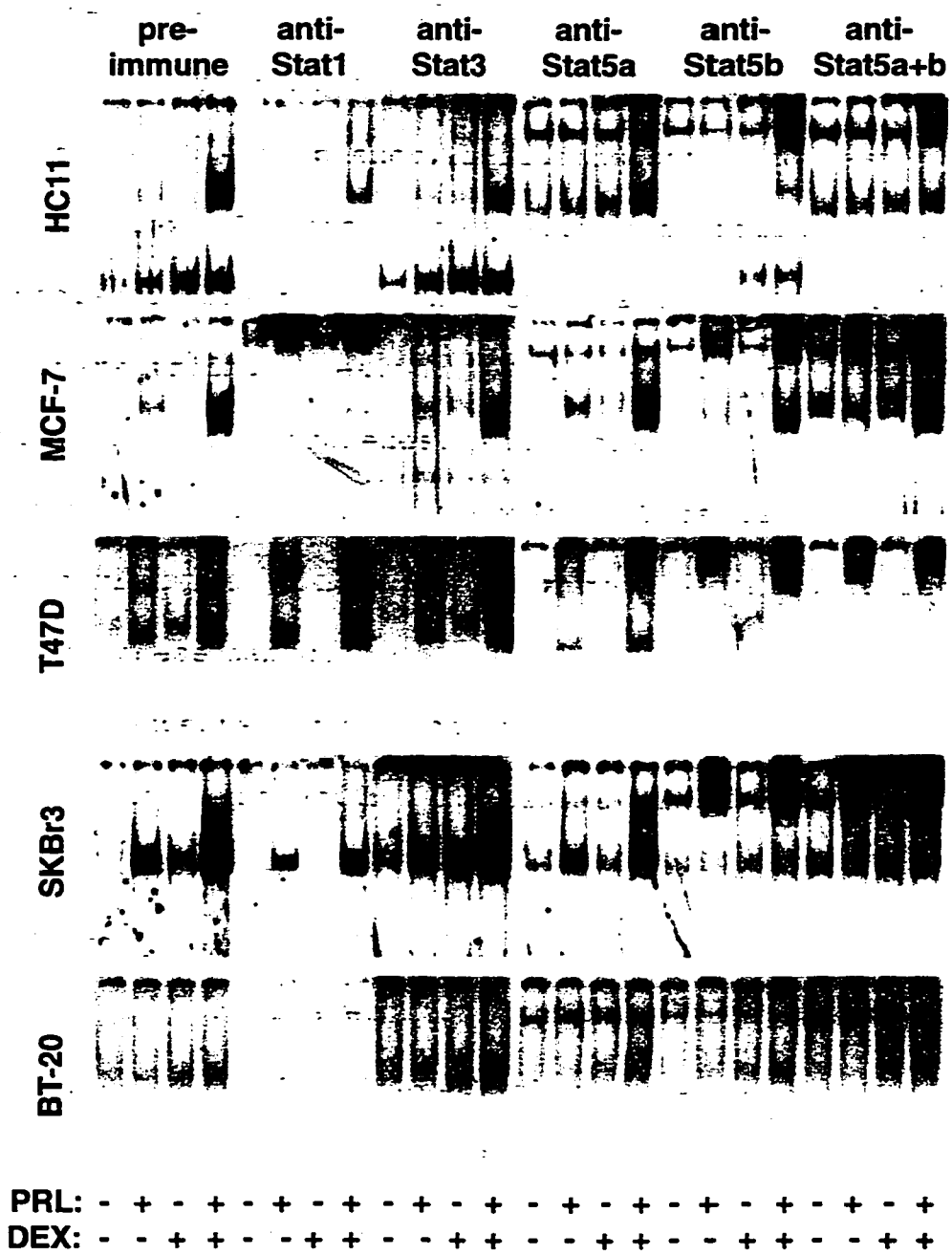
Interestingly, however, a moderate level of constitutive tyrosine phosphorylation of STAT5b was present in the SKBr3 cells (fig 4B, panel 4, lane c).

Upregulation of prolactin-induced STAT5 DNA binding

Dexamethasone upregulates prolactin-induced STAT5 DNA binding to the beta-casein promoter in human breast cancer cell lines. Although the positive effect of dexamethasone pretreatment on prolactin-induced STAT5 tyrosine phosphorylation levels was most pronounced in T47D cells, EMSA analyses showed that dexamethasone synergized with prolactin at the level of STAT-DNA binding in each of the cell lines examined (fig. 12). However, there was significant variability between the cancer cell types, both quantitatively and qualitatively.

In this figure, the first four lanes represent standard conditions using cell extracts that have been incubated in the presence of control preimmune serum. The subsequent five sets of four lanes show samples that have been incubated with antibodies to either STAT1, STAT3, STAT5a, STAT5b, or STAT5a and STAT5b. These various antibodies will selectively remove or "supershift" DNA complexes containing the target STAT protein.

Fig. 12. Electrophoretic mobility shift assay (EMSA). Dexamethasone upregulates prolactin-induced STAT5 DNA binding to the β-casein promoter in human breast cancer cell lines. Quiescent breast cancer cells were maintained in serum-free medium for four days with or without dexamethasone (DEX; 1 μM), then stimulated for 15 min with prolactin (PRL; 10 nM), and lysates corresponding to 10 μg of protein were incubated either with normal rabbit serum, anti-STAT1, anti-STAT3, anti-STAT5a, anti-STAT5b, or anti-STAT5a and b serum in combination with 1 ng of [^{32}P]-labeled oligonucleotide probe corresponding to the PRL response element of the rat β-casein gene.


pre-immune
anti-Stat1
anti-Stat3
anti-Stat5a
anti-Stat5b
anti-Stat5a+b
HC11
MCF-7
T47D
SKBr3
BT-20
PRL: - + - + - + - + - + - + - + - + - + - + - + - +
DEX: - - + + - - + + - - + + - - + + - - + + - - + +

Previous analysis of prolactin-induced STAT5 binding to the beta-casein promoter by EMSA has revealed the existence of two STAT5-containing complexes (Kirken, et al., 1997a; Schaber, et al., 1998). Similar growth hormone-induced complexes have been designated complex I and a slower-migrating complex II (Waxman, et al., 1995; Ram, et al., 1996). Adopting this nomenclature, in HC11 cells, prolactin alone induced a STAT5 complex I that was markedly enhanced by dexamethasone pretreatment (fig. 12). In these cells, only low levels of STAT5 complex II were induced. In MCF-7 cells prolactin induced a STAT5 complex I, which was enhanced by dexamethasone pretreatment. However, dexamethasone also caused formation of a comparably strong STAT1 complex in MCF-7 cells. In T47D cells, prolactin induced equally strong STAT5 complexes I and II, both of which were enhanced by dexamethasone pretreatment. In SKBr3 cells prolactin induced a strong STAT5 complex I, and dexamethasone pretreatment increased this as well as leading to the formation of a complex II. In BT-20 cells, only a weak constitutive complex was observed that was enhanced by dexamethasone. This STAT-DNA complex in BT-20 cells was completely eliminated by incubation with anti-STAT1 serum, but was not affected by antisera to other STATs, suggesting that it consisted

primarily of activated STAT1.

In general, the antibody supershift data obtained in these studies reflect a high degree of complexity and combinatorial possibilities for STAT binding to the beta-casein promoter. It can be concluded from these observations that the extent of cell-dependent differences in prolactin and dexamethasone-induced responses should caution against simplistic models and interpretations of STAT binding data, particularly if based on data from single cancer cell lines.

In order to summarize the supershift data we have made the following three sets of observations:

1) Antibody-induced STAT5 homodimer formation. Antibodies to the carboxy-termini of STAT5a or STAT5b induce by themselves slow-migrating STAT5 complexes with the beta-casein promoter in a prolactin-independent manner. These high-molecular weight complexes are seen particularly well in HC11, MCF-7, SKBr3 and BT-20 cells, and less well in T47D cells. We postulate that formation of homodimers of unactivated STAT5a or STAT5b is induced by these bivalent antibodies, and that these antibody-induced homodimers are able to bind to the DNA in a manner similar to prolactin-activated dimers. In contrast, antibodies to STAT1 or STAT3 do not form homodimers that bind to the beta-casein probe.

2) STAT5a and STAT5b homo- and heterodimers coexist. With the exception of BT-20 cells, antibodies to STAT5b effectively supershifted significant amounts of prolactin-induced STAT-DNA complexes, including both the fast-migrating STAT5-complex I and the slower-migrating complex-II. Antibodies to STAT5a, on the other hand, were less effective in supershifting, leaving what we assume are STAT5b-containing dimer complexes. Antibodies to STAT5a were most efficient in dexamethasone-treated T47D cells and HC11 cells, which correlates with observed tyrosine phosphorylation of STAT5a in these cells.

3) Unexpected induction of STAT5-STAT1 heterodimers. In MCF-7 cells, prolactin induced a fast-migrating STAT5-containing complex I. This complex was enhanced by dexamethasone pretreatment, which also led to the formation of a second, even faster-migrating prolactin-induced complex. Consistent with its migration as a STAT1 homodimer complex (Kirken, et al., 1997a; Schaber, et al., 1998), this faster-migrating, dexamethasone-dependent complex in MCF-7 cells was completely supershifted by anti-STAT1 antibodies but not by antibodies to STAT3 or STAT5. More intriguingly, in MCF-7 cells, antibodies to STAT1 also depleted significant levels of STAT5-containing complexes. A similar effect of anti-STAT1

antibodies on STAT5-containing complexes was seen in HC11 and SKBr3 cells, but not in T47D cells. This effect of anti-STAT1 serum was specific, since anti-STAT3 antibodies had no effect. This raises the serious possibility that in certain cells prolactin can induce formation of STAT5-STAT1 heterodimers. In previous studies of hematopoietic cells and T47D mammary cells we have only observed prolactin-induced STAT1 homodimers (Kirken. et al., 1997a; Schaber, et al., 1998). The function of these putative STAT1-STAT5 heterodimers is completely unknown, but significantly increases the combinatorial possibilities for transcriptional control by prolactin. An alternative, but equally intriguing interpretation, is that antibodies to STAT1 depletes a shared component used by both STAT5 and STAT1 to form DNA-binding complexes. A candidate such component would be p48, a Myb-related protein which stabilizes STAT1-STAT2 heterodimers (Darnell, et al., 1994).

Finally, it should be emphasized that the positive interaction between glucocorticoids and prolactin at the level of phosphorylation of STAT5 proteins and at the DNA-binding level, is further compounded at the transcriptional level given the recent description of a direct role of the glucocorticoid receptor as a ligand-dependent coactivator of

STAT5 transcription factors (Stoeklin, et al., 1997).

DISCUSSION

The present study provides novel evidence that glucocorticoids can regulate prolactin activation of STAT5a and thus modulate the extent of prolactin-induced differentiation of mammary cancer cells. Pretreatment of the human breast cancer cell line T47D with dexamethasone for 2-4 days was required for prolactin to activate STAT5a as evidenced by a marked upregulation of prolactin-induced STAT5a tyrosine phosphorylation and STAT5a-STAT5b heterodimer formation. This modulation of prolactin signal transduction correlated with induction of differentiation markers casein and PIP. A similar but less marked stimulation by dexamethasone pretreatment of prolactin-activated STAT5a was seen in MCF-7 cells. Furthermore, STAT5a expression was lost in the undifferentiated, ER-negative BT-20 and SKBr3 cell lines and could not be rescued by dexamethasone treatment. In contrast, the well-differentiated, near-normal mammary cell lines MCF-10A and HC11 expressed comparably high levels of both STAT5a and STAT5b. These studies therefore identify STAT5a as a target for dexamethasone regulation of prolactin

signal transduction, a finding that has potential importance for differentiation therapy of mammary cancers, and implicate STAT5a as a candidate differentiation marker of possible prognostic importance for human breast cancer progression.

Prolactin has been shown to activate several members of the STAT transcription factor family, including STAT1, STAT3, STAT5a and STAT5b (Gouilleux, et al., 1994; David, et al., 1994; DaSilva, et al., 1996; Schaber, et al., 1998). Of these, STAT5a and STAT5b bind strongly to the prolactin response element of the beta-casein gene, whereas STAT1 and STAT3 show little or no ability to bind to the promoter region of this milk protein gene (Schaber, et al., 1998; Kirken, et al., 1997a). Although both STAT5a and STAT5b bind to and activate transcription of beta-casein reporter genes in COS-7 cells (Doppler, et al., 1989; Schmitt-Ney, et al., 1991; Happ, et al., 1993), STAT5a has been thought to be particularly important for mammary gland differentiation. STAT5a-deficient female mice have a severe phenotypic loss of prolactin-induced milk production (Liu, et al., 1997), whereas the phenotype of corresponding STAT5b knockout mice is characterized by specific growth hormone signaling defects, resulting in stunted growth and liver dysfunction with little impact on milk production (Udy, et al., 1997).

The finding in the present study that prolactin activation of STAT5a is regulated by dexamethasone in some, but not all human breast cancers, has direct implications for differentiation therapy of human breast cancer. The data suggest that in normal cells, STAT5a is operable and available to prolactin receptors, in moderately differentiated cancer cell lines (MCF-7, T47D), dexamethasone treatment stimulates the extent of prolactin induced STAT5a activation, whereas the more malignant SKBr3 and BT-20 are refractory to dexamethasone treatment, at least with regard to prolactin induced tyrosine phosphorylation of STAT5a. The current observations provide incentive for more general and systematic screening efforts of breast tumors for STAT5 activation patterns. These findings also stimulate, in order to understand the involvement of prolactin in the development of mammary gland neoplasia, reconsideration of the repeated biologic evidence that prolactin can serve either as a mammary epithelium growth promoter on the one hand, or as a cytostatic differentiation agent on the other (Shiu, et al., 1984; Rosen, et al., 1994; Fuh, et al., 1995).

This dual capacity of prolactin to induce either cellular proliferation or differentiation of mammary target cells suggests the presence of thus far uncharacterized

physiological regulatory mechanisms that can control response switching at the cellular level. Implicit in this notion, that prolactin-induced responses are dependent on physiological conditions (e.g. cell specific factors, hormones or developmental maturation) is the concept that prolactin might function as tumor promoter only when the physiological environment or pathophysiologic changes favor prolactin receptor-mediated growth. Indeed, the prolonged controversy over the involvement of prolactin in human breast cancer etiology and progression might to a large extent be explained if circulating or autocrine prolactin proved to serve as a conditional mammary tumor promoter. A duality of prolactin actions might also illuminate why multiparity and prolonged suckling tend to lower breast cancer risk (Kalache, et al., 1993). It would be reasonable to hypothesize that perinatal physiological conditions might yield a protective effect by fostering the differentiating effects of prolactin. Thus, a better understanding of how prolactin acts as a conditional growth factor or tumor promoter, or conversely as a differentiation agent, becomes a critical issue with strong relevance to the problem of growth factor-induced breast cancer development and growth factor-based therapeutic strategies.

Similar dual roles as context-dependent growth or differentiation agents have been established for a variety of the other four-helix bundle polypeptide cytokines and hormones that activate JAK-STAT pathways via a related family of cell surface receptors, including oncostatin M, leukemia-inhibitory factor, erythropoietin, and a majority of the interleukins (Schindler, et al., 1995).

To what extent transcriptional regulation of STAT5-responsive genes varies with STAT5a and STAT5b dimerization patterns remains unknown. The two proteins differ most in the COOH-terminal regions which are involved in docking of SH2 domains and transactivation. STAT5a and STAT5b can undergo homo- and heterodimerization, and bind to similar DNA response elements (Kirken, et al., 1997a). However, STAT5a and STAT5b may also form different complexes to DNA as revealed by EMSA (Kirken, et al., 1997a). The two transcription factors may therefore have both overlapping and distinct functions. Consistent with this notion, the immune functions of STAT5a and STAT5b are maintained in mice deficient in either gene (Liu, et al., 1997; Udy, et al., 1997). However, STAT5b is unable to compensate for STAT5a-deficiency in female mice, resulting in phenotypic loss of prolactin-induced milk production (Liu, et al., 1997).

Conversely, STAT5b knockout mice have specific growth hormone signaling defects, resulting in stunted growth and liver dysfunction (Udy, et al., 1997). Other differences between STAT5a and STAT5b are demonstrated by differing degrees of serine phosphorylation in interleukin-2 stimulated lymphocytes (Kirken, 1997b). We have also found that STAT5a was constitutively phosphorylated on serine to a significantly higher extent than STAT5b was when expressed in COS-7 cells (Yamashita, et al., submitted). Therefore, a series of dissimilarities points toward unique control mechanisms and possibly specialized functions of STAT5a and STAT5b proteins. Systematic testing of STAT5-regulated genes other than beta-casein is needed to clarify this issue.

Regarding the role of STAT5 transcription factors in breast cancer, it is of particular interest to note that the STAT5a and STAT5b genes are localized to chromosome 17q11.2 (Lin, et al., 1996). Markers mapping to this locus reveal loss of heterozygosity in 25-79% of human breast cancers (Futreal, et al., 1992) consistent with the presence of tumor suppressor gene(s). The recently established BRCA1 tumor suppressor gene is also localized to this arm of chromosome 17 (Black, et al., 1993). Future studies will specifically examine the frequency of loss of STAT5 gene expression in

human breast cancer.

In summary, we present novel evidence that glucocorticoids can regulate prolactin activation of STAT5a and thus modulate the extent of prolactin-induced differentiation of mammary cancer cells. Furthermore, STAT5a expression was lost in the undifferentiated, ER-negative BT-20 and SKBr3 cell lines and could not be rescued by dexamethasone treatment, whereas well-differentiated, near-normal mammary cell lines MCF-10A and HC11 expressed comparably high levels of both STAT5a and STAT5b. These studies therefore implicate STAT5a as a critical differentiation factor of potential importance for glucocorticoid therapy of certain mammary cancers.

INDEPENDENT STAT1 SIGNALING BY PRL AND INTERFERONS

Introduction

Whereas the type I interferons (interferon-alpha and interferon-beta) preferentially induce heterodimers of STAT1 with either STAT2 or STAT3 (Beadling, et al., 1994; Ghislain, et al., 1996), prolactin appears to primarily activate STAT1 as homodimers (Kirken and Grimley, 1997). In addition, prolactin is capable of activating STAT3 and STAT5 isoforms in responsive cells (Kirken and Grimley, 1997; DaSilva, et al., 1996). Prolactin is especially relevant as a potential type I interferon-antagonist in breast cancer progression because 40-70% of breast tumors express prolactin receptors (Bonneterre, et al., 1990; Murphy, et al., 1984). Furthermore, an effect of prolactin as an autocrine growth factor in human breast cancer cells may also be significant (Ginsburg, et al., 1995; Clevenger, et al., 1995). Prolactin receptors are also potently activated by human growth hormone (Somers, et al., 1994). As stress hormones, both growth hormone and prolactin may become elevated in cancer patients (van der Pompe, et al., 1996).

The principal aim of the present study was to examine whether prolactin would interfere with type I interferon signal transduction by competing for limited cytoplasmic STAT factors, thus possibly antagonizing the antiproliferative effect of type I interferons in breast cancer treatment. We were particularly interested in determining the extent of signal specificity between prolactin and the type I interferons at the level of STAT1. A second goal of relevance to the efficacy of type I interferons in breast cancer therapy was to test if mammary tumor cell lines could become sensitized to interferon-alpha and -beta by pretreatment with the type II interferon, interferon-gamma, an effect that has been observed in other epithelial cell lines (Fleischmann, et al., 1984).

Analysis of the effect of prolactin on type I interferon-induced tyrosine phosphorylation of STAT proteins and their binding to response elements of a series of interferon-regulated genes, including the interferon-stimulated gene 15 (ISG15), interferon regulatory factor-1 (IRF1), and the Fcγ receptor, now shows that costimulation of prolactin receptors did not interfere with type I interferon signals in several human breast cancer cell lines, including T47D, MCF-7 and BT-20. Despite significant overlap in the use

of STATs by these interferons and prolactin, the results indicated a high degree of signaling specificity between the two receptor systems, and that cytoplasmic levels of the shared STAT1 and STAT3 proteins were not limiting, as evidenced by additive activation in the presence of both prolactin and the type I interferons. Similarly, prolactin did not interfere with interferon-induced growth inhibition. The results therefore do not support the notion that circulating or autocrine prolactin may antagonize antiproliferative type I interferon signals during adjuvant treatment of breast cancer. On the other hand, the study indicated that pretreatment of human breast cancer cell lines with interferon-gamma enhanced type I interferon signals and growth inhibition, suggesting a possible clinical approach for improving the efficacy of type I interferons in the treatment of breast cancer patients.

RESULTS

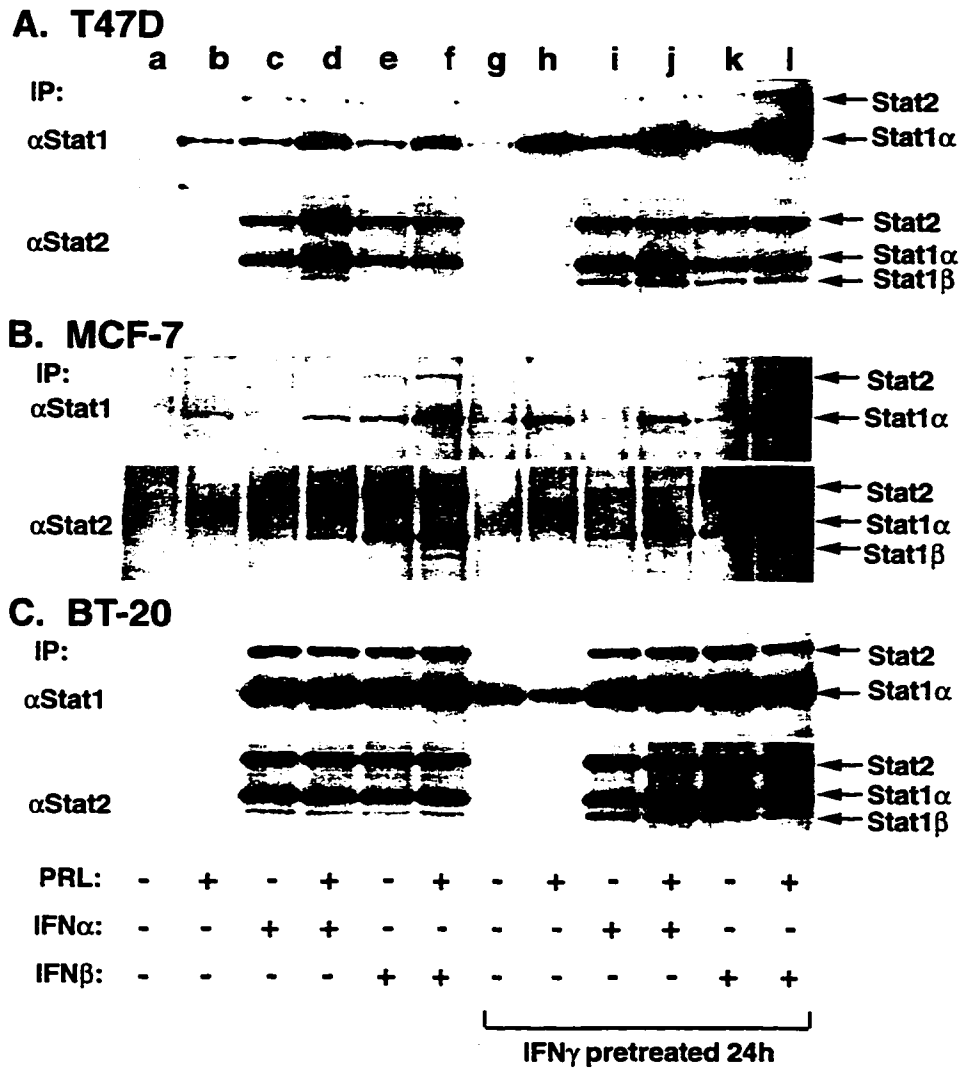
Induction of STAT1/STAT2 dimers in human mammary cell lines.

Prolactin activation of STAT1 does not interfere with interferon induction of tyrosine phosphorylated STAT1/STAT2 heterodimers in human mammary cell lines. To test the

influence of prolactin on STAT signals induced by type I interferons, we first examined the effect of cotreatment of prolactin and interferon on STAT1 and STAT2 tyrosine phosphorylation. Three different human cell lines derived from mammary adenocarcinomas, including T47D, MCF-7, and BT-20, were tested for inducible tyrosine phosphorylation of STAT1 and STAT2 (fig. 13). Exponentially growing, subconfluent cells that had been incubated in medium without fetal calf serum for 24 h to reduce background tyrosine kinase activities, were stimulated for 15 min with either prolactin, interferon-alpha, interferon-beta, or a combination of either interferon and prolactin. STAT1 or STAT2 were immunoprecipitated with specific antisera from parallel samples of cleared cell lysates and analyzed for tyrosine phosphorylation by immunoblotting.

In general, coactivation of prolactin receptors did not interfere with the ability of interferon receptors to induce phosphorylation of either STAT1 or STAT2 (fig. 13A-C, lanes a-f), demonstrating that the cytoplasmic levels of the shared STAT1 protein are not limiting. In T47D cells, prolactin alone stimulated tyrosine phosphorylation of the 91 kDa STAT1α to an extent comparable to that induced by interferon-alpha or interferon-beta (fig. 13A, upper panel, lanes b, c,

Fig. 13. Immunoblot analysis. Prolactin (PRL) activation of STAT1 does not antagonize type I interferon (interferon-alpha and interferon-beta) induction of tyrosine phosphorylated STAT1/STAT2 heterodimers in human mammary cell lines. Quiescent breast cancer cells T47D, MCF-7 and BT-20 were incubated for 15 min at 37°C with prolactin (PRL; 20 nM), interferon-alpha (IFNα; 1000 U/ml), and/or IFNβ (1000 U/ml) as indicated (lanes a-f), and lysates immunoprecipitated (IP) with anti-(α)STAT1 (upper panels) or αSTAT2 sera (lower panels). Samples were separated by SDS-PAGE, transferred to a PVDF membrane, and immunoblotted with antiphosphotyrosine (α-PY) mAbs. Results were visualized by enhanced chemiluminescence. Note that pretreatment of human breast cancer cell lines with interferon-gamma (IFNγ; 10 ng/ml; 24h) enhanced type I IFN-induced formation of STAT1β/STAT2 heterodimers.


A. T47D
a b c d e f g h i j k l
IP:
αStat1
αStat2
Stat2
Stat1α
Stat1β
B. MCF-7
IP:
αStat1
αStat2
Stat2
Stat1α
Stat1β
C. BT-20
IP:
αStat1
αStat2
Stat2
Stat1α
Stat1β
PRL: - + - + - + - + - + - +
IFNα: - - + + - - - - + + - -
IFNβ: - - - - + + - - - - + +
IFNγ pretreated 24h

e). The effect on STAT1 tyrosine phosphorylation was additive when prolactin was administered in combination with either interferon-alpha or interferon-beta (fig. 13A, lanes d,f). Prolactin cotreatment also did not interfere with interferon-alpha- or interferon-beta-induction of STAT2 tyrosine phosphorylation or STAT1/STAT2 heterodimer formation, as judged by the levels of tyrosine phosphorylated STAT2 that coprecipitated with STAT1 in interferon-alpha- or interferon-beta-treated samples (fig. 13A, upper panel, lanes c-f), and the levels of STAT1 coimmunoprecipitating with STAT2 (fig. 13A, lower panel, lanes c-f). The antiserum against STAT2 was more efficient at coimmunoprecipitating STAT1 than vice versa, but there were no indications that prolactin interfered with STAT1-STAT2 dimerization by interferons. Similar results were observed in MCF-7 cells (fig. 13B, lanes a-f), although these cells were less responsive to interferon-alpha than to interferon-beta. In BT-20 cells, prolactin also did not interfere with interferon signaling via STAT1/STAT2 proteins (fig. 13C, upper panel, lanes a-f). However, in BT-20 cells the prolactin-induced STAT responses were consistently weak for as yet unclear reasons. These cells are known to express significant levels of prolactin receptors, approximately 8,000 per cell (Shui, et al., 1979),

but consistent with diminished STAT responses, we have also detected only weak prolactin-inducible activation of the JAK2 tyrosine kinase (Rui, et al., submitted).

Collectively, the results showed that prolactin receptor stimulation does not interfere with the ability of type I interferons to signal via STAT1 or STAT2. Although both prolactin and type I interferons used STAT1, there was no indication that cellular STAT1 levels were limiting for the two receptor systems.

Pretreatment of breast cancer cells with interferon-gamma.

Pretreatment of human breast cancer cell lines with interferon-gamma enhanced type I interferon-induced formation of STAT1β/STAT2 heterodimers. Pretreatment with interferon-gamma has been shown to enhance type I interferon responses in other epithelial cells (Fleischmann, et al., 1984). We therefore tested the effect of interferon-gamma pretreatment on STAT1 and STAT2 activation by type I interferons and prolactin in the three human mammary cell lines (fig. 13A-C, lanes g-l). Particularly in T47D and BT-20 cells, interferon-gamma pretreatment stimulated type I interferon-induced association of the 84 kDa STAT1β isoform with STAT2 (fig. 13A and C, lower panels, lanes i-l). This shorter STAT1β isoform

is a COOH-terminally truncated, alternatively spliced STAT1 variant without transactivating capacity (Bromberg, et al., 1996). However, too little is currently known about the function of STAT isoforms to predict how such a selective increase in STAT1β/STAT2 heterodimers might influence the biological activities of type I interferons in mammary epithelial cells. Interestingly, interferon-gamma-pretreatment enhanced prolactin-induced STAT1α activation in the T47D and MCF-7 lines (fig. 13A and B, lanes b and h). However, this increased use of STAT1α by prolactin again did not alter the ability of interferon-alpha or interferon-beta to activate STAT1α, as seen by additive phosphorylation responses (fig. 13A, upper panel, lanes h, j, l). Thus, consistent with the previously observed lack of interference by prolactin on type I interferon signals, similar signal independence was observed in interferon-gamma-pretreated cells.

Prolactin costimulation does not antagonize IFNα/β-activation.

Prolactin costimulation does not antagonize type I interferon-activation of other interferon-responsive STATs in human mammary cell lines. In addition to sharing the signaling component STAT1, both prolactin and the type I

interferons have been reported to employ STAT3 and STAT5 proteins in their signaling pathways (DaSilva, et al., 1996; Meinke, et al., 1996). To test whether prolactin interfered with the ability of the type I interferons to signal via these potentially overlapping STATs, we examined their regulated tyrosine phosphorylation states in the same panel of human breast cancer cells. Because interferon-beta had been shown to be the most potent general inducer of STAT activation in the three breast cancer cell lines (Coradini, et al., 1994) (fig. 13), we selected this type I interferon for use in subsequent experiments. After 24 h of incubation in serum-free medium, the cells were exposed for 15 min to either prolactin or interferon-beta alone, or a combination of the two, as described previously.

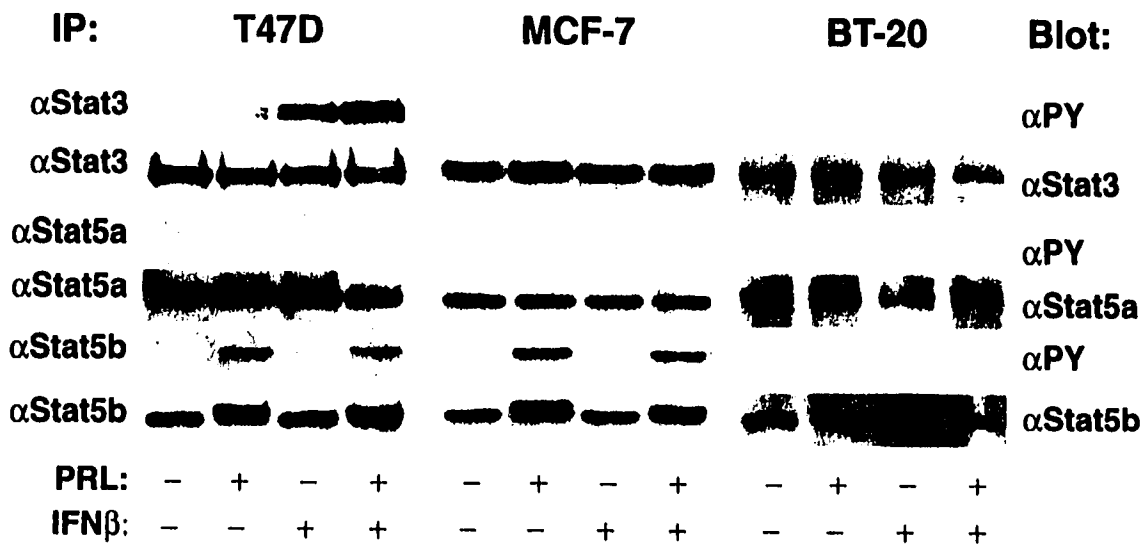
Corresponding to our observations with STAT1 and STAT2 signaling, there were no indications that prolactin receptor activation interfered with the ability of type I interferons to induce tyrosine phosphorylation of STAT3, STAT5a or STAT5b (fig. 14). Curiously, despite plentiful expression both in MCF-7 and BT-20 cells, significant and consistent activation of STAT3 by both interferon-beta and prolactin was detected only in T47D cells. As shown for STAT1 (fig. 13), cotreatment with prolactin and interferon resulted in a simple additive

effect on STAT3 tyrosine phosphorylation was observed when prolactin was coadministered with interferon-beta to T47D cells (fig. 14, column 1, panel 1).

In analogous manner, although both STAT5a and STAT5b were expressed in all cell lines, interferon-beta did not induce tyrosine phosphorylation of either STAT5 isoform. Prolactin, on the other hand, induced marked but selective tyrosine phosphorylation of STAT5b and not of STAT5a, in MCF-7 and T47D cells (fig. 14, columns 1 and 2, panels 5 and 6). The lack of prolactin-induced STAT5a activation in these cells was unexpected in light of the proven ability of prolactin receptors to recruit STAT5a in other cells including mammary cells (Schmitt-Ney, et al., 1991), and the dramatic effect STAT5a deficiency has on the mammary gland lactational phenotype (Liu, et al., 1997). This apparent inability of prolactin receptors to activate STAT5a in T47D and MCF-7 cells may reflect a molecular defect or indicate that STAT5 isoform selection by prolactin receptors involves a regulated mechanism.

In contrast to the enhancement by interferon-gamma pretreatment of type I interferon-induced STAT1β recruitment, interferon-gamma did not modulate any of the responses of STAT3 or STAT5 in the three cell lines (data not shown).

Fig. 14. Immunoblot analysis. Prolactin cotreatment does not antagonize type I interferon (interferon-alpha and interferon-beta) activation of other IFN-responsive STATs in human mammary cell lines. Quiescent breast cancer cells were incubated for 15 min at 37°C with prolactin (PRL; 20 nM), interferon-beta (IFNβ; 1000 U/ml), or a combination, as indicated, and lysates immunoprecipitated (IP) with anti-(α)STAT3 (upper panels), αSTAT5a (middle panels), or αSTAT5b (lower panels). Samples were separated by SDS-PAGE, transferred to PVDF, and immunoblotted with either antiphosphotyrosine (α-PY) mAbs or the same Ab used for immunoprecipitation. Results were visualized by enhanced chemiluminescence.


IP:
T47D
MCF-7
BT-20
Blot:
αStat3
αStat3
αStat5a
αStat5a
αStat5b
αStat5b
αPY
αStat3
αPY
αStat5a
αPY
αStat5b
PRL: − + − + − + − + − + − +
IFNβ: − − + + − − + + − − + +

Interferon-induced and prolactin-induced STAT-DNA complexes.

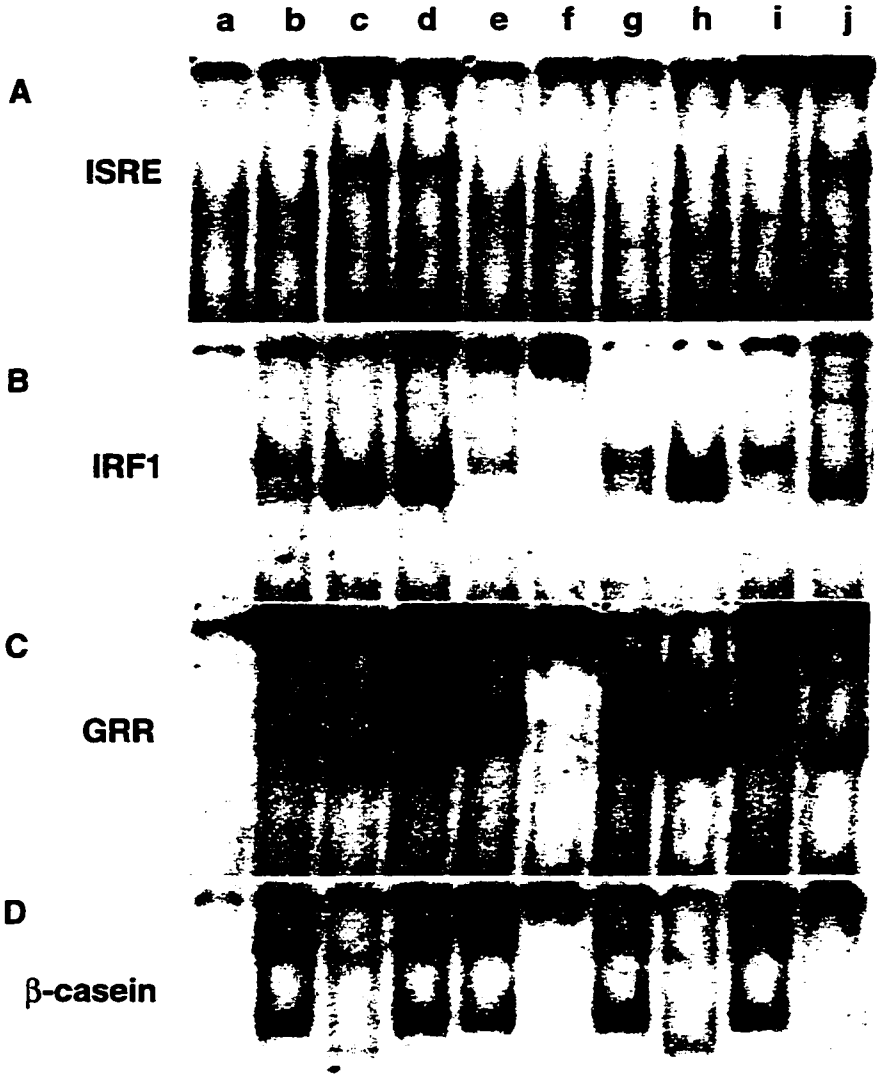
Interferon-induced STAT-DNA complexes are independent and qualitatively different from prolactin-induced STAT-DNA complexes. An important question remained as to whether prolactin competed with interferon-induced STAT signals at the level of DNA-binding. We analyzed the inducible formation of STAT-DNA complexes by electrophoretic mobility shift assays, using probes derived from several known interferon-responsive genes. Because T47D cells exhibited the strongest combined prolactin and interferon-beta responses at the STAT tyrosine phosphorylation level among the three cell lines tested, nuclear extracts from these cells were used to examine inducible STAT binding to the interferon-response elements of the ISG15 gene, IRF1 and Fcγ receptor. We also analyzed STAT complex formation with the prolactin-response element of the beta-casein gene.

The electrophoretic mobility shift analyses revealed marked differences between interferon-beta- and prolactin-induced complexes, and showed also at this level that when administered simultaneously, prolactin did not interfere with interferon-beta-induced signals. Specifically, prolactin did not induce any complex with the ISRE of the ISG15 gene, and did not disrupt the ability of interferon-beta to induce STAT

binding to this response element (fig. 15A, lanes a-d). This interferon-beta-induced STAT-ISRE complex was completely supershifted with antibodies to either STAT1 or STAT2, but not to STAT3 (fig. 15A, lanes f, h, and j), which is consistent with binding of interferon-induced STAT1-STAT2 heterodimers. The ISG15 gene encodes an immunomodulatory cytokine and is possibly involved in the antiproliferative effect of interferons (D' Cunha, et al., 1996).

Both prolactin and interferon-beta induced STAT binding to the GAS site of the IRF1 gene promoter (fig. 15B). IRF1 is a tumor suppressor gene known to be activated by both interferon-beta and prolactin (Yu-Lee, et al., 1990; Wang, et al., 1997). However, prolactin-induced IRF1 complexes differed from those induced by interferon-beta. In T47D cells, prolactin induced two separate complexes with the IRF1 sequence, a predominant slow migrating complex and a weaker fast migrating complex (fig. 15B, lane b). Consistent with it constituting a STAT1 complex, the weaker prolactin-induced band was specifically supershifted by anti-STAT1 serum, but not with antisera to STAT2 or STAT3 (fig. 15B, lanes e, g, and i). The predominant, slow-migrating prolactin-induced complex could be efficiently supershifted with anti-STAT5b serum, but not with antiserum to STAT5a (data not shown), and

Fig. 15. Electrophoretic mobility shift assay (EMSA). IFN-induced STAT-DNA complexes are independent and qualitatively different from PRL-induced STAT-DNA complexes. Quiescent T47D cells were incubated with interferon-beta (IFNβ; 1000 U/ml) and/or prolactin (PRL; 20 nM), as indicated, for 10 min at 37°C, and lysates corresponding to 10 μg of protein were incubated either with normal rabbit serum (lanes a-d), anti-(α)STAT1 (lanes e, f), αSTAT2 (lanes g, h), or αSTAT3 serum (lanes i, j) in combination with 1 ng of [^{32}P]-labeled oligonucleotide probe corresponding to the interferon-stimulated response element (ISRE) of ISG15, the IRF-1 gene promoter, the GRR response element of the FcγR1 gene, or the PRL response element of the rat β-casein gene.



PRL	-	+	-	+	+	-	+	-	+	-
IFNβ	-	-	+	+	-	+	-	+	-	+
α-STAT					1	1	2	2	3	3

corresponded in size to the STAT5 complex induced by prolactin in rat Nb2 cells (Kirken and Grimley, 1997). This is consistent with the observed exclusive tyrosine phosphorylation of STAT5b and not STAT5a by prolactin in T47D cells (fig. 14), and we infer that this complex contains STAT5b homodimers. interferon-beta, in contrast, induced marked formation of only one fast-migrating complex, which was completely supershifted by anti-STAT1 serum, and also to a significant extent by anti-STAT3 serum (fig. 15B, lanes c, f, and j). These data suggest that the interferon-beta-induced IRF1 complex consisted predominantly of STAT1-STAT3 heterodimers, and thus differed qualitatively from the two prolactin-induced complexes.

When we analyzed prolactin- and interferon-beta-induced STAT binding to the GRR response element of the FcγR1 gene, another interferon-responsive gene (fig. 15C, lanes a-j), results were highly correspondent to those obtained with the IRF1 probes. This further suggested independence of action by prolactin and interferon signaling pathways, with no evidence for prolactin interfering with interferon signals. Additional evidence for independence of signals from interferon and prolactin receptors was provided by differences in prolactin and interferon-beta-induced complexes with the GAS sequence

of the beta-casein gene promoter. The beta-casein gene is a known STAT5-regulated gene (Liu, et al., 1995), and the beta-casein-derived GAS sequence formed two distinct prolactin-induced complexes which corresponded to two prolactin-inducible, STAT5-containing DNA complexes observed in Nb2 cells (Kirken and Grimley, 1997; Rui, et al., 1998). Consistent with this notion, antisera useful for supershift assays of complexes containing human STAT1, STAT2 or STAT3 proteins did not shift either of these two complexes (fig. 15D, lanes e, g, and i), and antisera directed against the COOH-terminus of mouse STAT5b, but not to STAT5a, could supershift this putative homodimeric STAT5b complex (data not shown). Each of these STAT5 antisera is also useful in supershift analysis of the corresponding human STAT5 isoforms (Kirken and Grimley, 1997; Kirken and Erwin, 1997). interferon-beta, on the other hand, caused the formation of two weak complexes that could be supershifted by either STAT1 and STAT3 sera, but not with STAT2 serum, suggesting that interferon-beta-induced heterocomplexes of STAT1 and STAT3 may interact only weakly with the beta-casein probe.

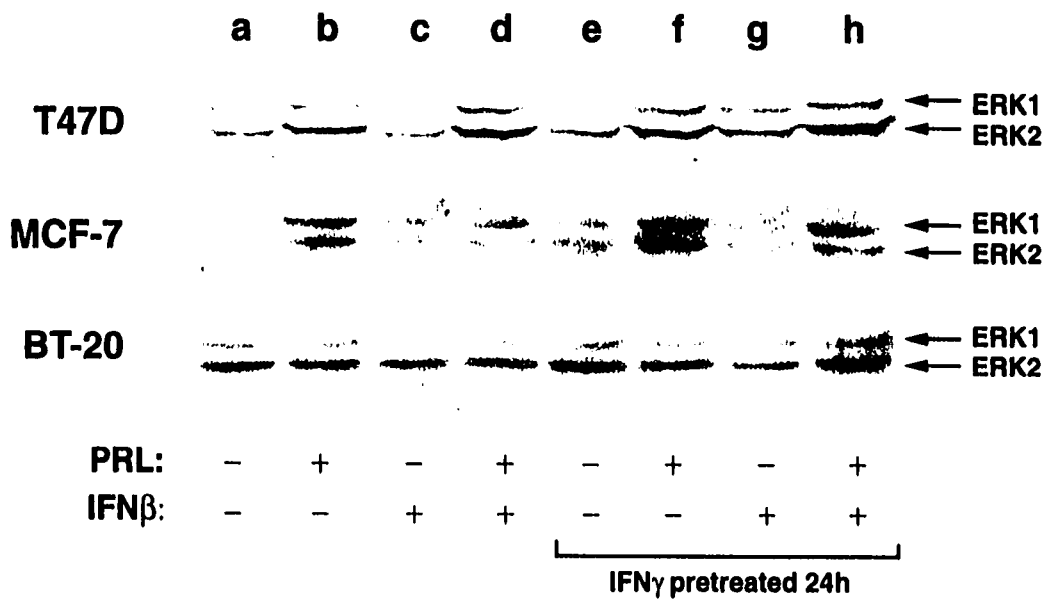
We conclude that the electrophoretic mobility shift assays further corroborated the notion that prolactin does not interfere with type I interferon receptor signals at the

level of STAT proteins. In particular, interferon-beta-induced STAT1-containing complexes were not affected by the parallel formation of distinct prolactin-induced STAT1 complexes, which differed with regard to DNA sequence selectivity and composition.

Independent activation of MAPK by prolactin and IFN-beta.

Activation of MAPK by prolactin is independent from interferon-beta signaling in human breast cancer cells. Mitogen activated protein kinases (MAPK) have been proposed to constitute regulated serine kinases critical for the full transactivation potential of STAT1 and STAT3 (Wen, et al., 1995; David, et al., 1995). Both prolactin and interferons have been demonstrated to stimulate MAPKs in various cells (David, et al., 1995; Carey, et al., 1995; Stancato, et al., 1997). We therefore also examined the potential for interpathway crosstalk between prolactin and interferon-beta at this level. Anti-active MAPK antibodies were used to assay for prolactin- and interferon-stimulation of MAPK (fig. 16). Despite marked STAT activation as presented above, there was no detectable stimulation of MAPKs by interferon-beta in the three breast cancer cell lines. Prolactin, on the other hand, activated the two MAPKs, ERK1 and ERK2, in MCF-7 cells and,

Fig. 16. Immunoblot analysis. Stimulation of mitogen activated protein kinases (MAPK) by PRL is independent from IFNβ signaling in human breast cancer cells. Quiescent breast cancer cells were incubated for 15 min at 37°C with prolactin (PRL; 20 nM) and/or interferon-beta (IFNβ; 1000 U/ml), as indicated (lanes a-d), and 50 μg of protein from whole cell lysates were separated by SDS-PAGE. After electrophoresis, samples were transferred to a PVDF membrane and immunoblotted with antibodies to activated MAPK. Results were visualized by enhanced chemiluminescence. Bands corresponding to extracellular regulated kinase 1 (ERK1) and ERK2 are indicated by arrows. Parallel samples (lanes e-f) were pretreated for 24 h with interferon-gamma (IFNγ; 10 ng/ml).


a b c d e f g h
T47D
MCF-7
BT-20
ERK1
ERK2
PRL: − + − + − + − +
IFNβ: − − + + − − + +
IFNγ pretreated 24h

to a lesser extent, in T47D cells (fig. 16, lanes b and f). In T47D and MCF-7 cells stimulated with both factors in combination, there was no evidence of cross-talk or interference, again supporting the notion of signal independence between prolactin and interferon signaling pathways. Significant levels of constitutive MAPK activation were observed in T47D and BT-20 cells.

The extent of involvement of MAPKs in STAT activation by interferons and other cytokines is not yet fully understood (Ihle, et al., 1996). Our results suggest that interferons are capable of activating STATs without stimulating MAPKs, but this may be due to constitutive activation of MAPKs in these mammary cells. More important in the context of the present study, we also found no evidence for interference by prolactin on interferon-induced signals at the level of MAPKs. Finally, pretreatment of either of the three cell lines with interferon-gamma for 24h had no significant effect on the MAPK activation patterns (lanes e-h).

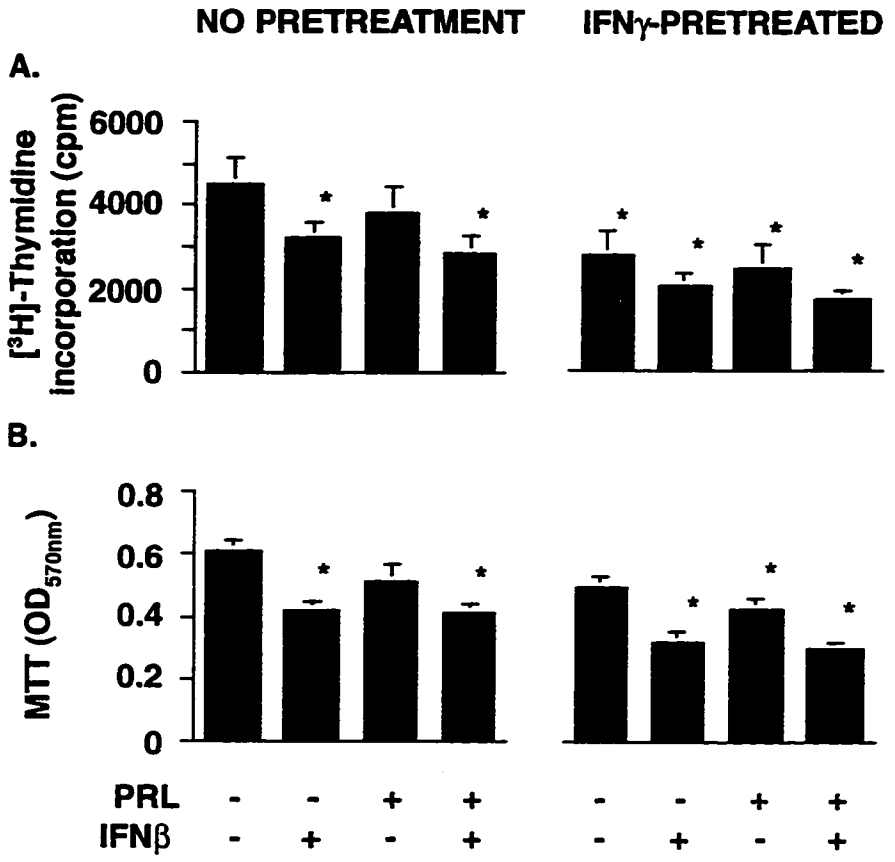
Prolactin does not mitigate anti-proliferative effect of IFNβ.

The antiproliferative effect of interferon-beta on breast cancer cell cultures is not mitigated by prolactin. Because the molecular analysis of STAT signal transduction

had suggested that prolactin and interferons maintain signal autonomy even while sharing several signaling components, we tested whether the antiproliferative effect of interferon-beta was also unaffected by cotreatment with prolactin. Exponentially growing T47D cells were cultured as described above with either prolactin, interferon-beta, or both, and subsequently pulsed with [^{3}H]-thymidine for 4 h. Incorporated thymidine was quantified and compared between the various treatments. Parallel cell cultures were also preincubated with low concentrations of interferon-gamma for 24 h to test whether this pretreatment could increase the efficacy of interferon-beta.

The results were consistent with a high degree of signal specificity and showed that prolactin cotreatment did not antagonize the antiproliferative effect of interferon-beta. The growth inhibitory effect of interferon-beta was approximately 30% of basal growth levels regardless of whether cells had been pretreated with interferon-gamma or not (fig. 17A). Under the culture conditions tested, prolactin alone did not significantly alter the proliferation rate, and did not affect the growth inhibition induced by interferon-beta. These results were corroborated by parallel experiments using MTT metabolic labeling in T47D cells (fig.

Fig. 17. Proliferation assays. The antiproliferative effect of IFNβ on breast cancer cell cultures is not mitigated by PRL. Mid-log phase T47D cells were treated 24 h with interferon-beta (IFNβ; 1000 U/ml) and/or prolactin (PRL; 10 nM), as indicated (panel A). Cells were pulsed 4 h with [^{3}H]-thymidine (10 μCi/ml), harvested onto glass fiber filters, and analyzed for [^{3}H]-thymidine uptake by liquid scintillation counting. Parallel samples were pretreated 24 h with interferon-gamma (IFNγ; 10 ng/ml) prior to hormone stimulation. Similar experiments were performed on T47D cells (panel B) using the MTT assay, as described in "Materials and Methods." Asterisks identify values which are statistically significant relative to controls ($p<0.05$), as determined by Sheffe's multiple range test after one-way ANOVA.


NO PRETREATMENT
IFNγ-PRETREATED
A.
[3H]-Thymidine incorporation (cpm)
6000
4000
2000
0
B.
MTT (OD570nm)
0.8
0.6
0.4
0.2
0
PRL - - + + - - + +
IFNβ - + - + - + - +

17B), again demonstrating that cotreatment with prolactin did not interfere with interferon-beta-induced growth inhibition. A similar relationship was observed in MCF-7 cells (data not shown). An unconstrained growth inhibition by interferon-beta in the presence of prolactin corresponded well with the observed lack of interference of prolactin on type I interferon-induced STAT signals. Of potential importance for the use of type I interferons in breast cancer treatment, we observed that pretreatment of breast cancer cultures with interferon-gamma for 24 h increased the overall growth inhibitory effect of interferon-beta from approximately 30% to 40-55%, depending on the the cell type and proliferation assay (fig. 17A, B; data not shown).

DISCUSSION

In summary, the present study suggests that competition for limited cytoplasmic STAT signaling proteins does not cause prolactin to interfere with normal signal transduction by the type I interferons. Consistent with a functional independence of type I interferon signals from prolactin signals, prolactin did not act as an antagonist of the antiproliferative effect of these type I interferons.

However, this study does not exclude the possibility that prolactin and other factors implicated in the progression of breast cancer may counteract the antiproliferative effect of interferons via independent signaling pathways. Further, given the observed cell-dependent differences in STAT expression and activation, examination of additional breast carcinomas may be required to establish the generality of our results. Collectively, our data provides no evidence for competition between the two signaling pathways for limiting STAT factors. Instead, the study indicated that prolactin and interferon receptors maintain an unexpectedly high degree of signal fidelity and specificity despite sharing signaling components. Finally, the data also suggest an enhancement of type I interferon responses by pretreatment of breast cancer cell cultures with interferon-gamma, a finding of possible importance for the use of type I interferons in the treatment of breast cancer patients.

SUMMARY

Endocrine therapy has proven a valuable approach to breast cancer. In particular, antiestrogens have demonstrated significant improvement in survival rates, and have recently been shown to prevent breast cancer development in women in high-risk populations. Other endocrine or cytokine-based therapies, including glucocorticoids and interferons which have been highly effective as adjuvant treatment of hematological cancers, have also shown promise in breast cancer. However, due to the less consistent clinical responses to both glucorticoids and interferons in breast cancer patients, research efforts have continued to focus on improving their efficiency.

Important recent insights into the underlying molecular biology of hormone signal transduction have identified the critical involvement of cytoplasmic STAT transcription factors. These molecular intermediaries convey the signal from the cell surface to the nucleus where they activate transcription of target genes. One hormone, which signals via the STAT pathway, is of particular importance in breast cancer: namely, the mammary growth and differentiation

factor, prolactin.

The specific aims of this study were: 1) to examine whether the glucocorticoid, dexamethasone, may promote the terminal differentiation of breast cancer cells by stimulating prolactin activation of the transcription factors, STAT5a and STAT5b; 2) to examine whether prolactin interferes with type I interferon signal transduction by competing for limited cytoplasmic STAT factors, thus antagonizing the antiproliferative effect of type I interferons in breast cancer treatment; and 3) to test if mammary tumor cell lines, like many hematopoietic cancer cells, become sensitized to the anti-proliferative effect of type I interferons by pretreatment with interferon-gamma.

After establishing differentiation conditions in breast cancer cells, STAT transcription factor expression, activation and DNA-binding were examined by immunoblot and electrophoretic mobility shift assay. Based on the research work presented in this thesis, we conclude that:

1) Glucocorticoids have a profound positive effect on prolactin signal transduction by STAT5 transcription factors in some, but not all breast cancer cells. STAT5a expression is clearly linked to differentiation of breast cancer cells, and our findings may have important implications for the use

of glucocorticoids in differentiation therapy of select breast cancer patients.

2) Prolactin activates STAT1 but does not disrupt STAT1-STAT2 heterodimer formation or the anti-proliferative effect of type I interferons in human breast cancer cells. In fact, cytoplasmic levels of STAT1 are not rate-limiting, and prolactin and type I interferons maintain an unexpectedly high degree of signal fidelity in human breast cancer cell lines despite activating overlapping sets of STAT transcription factors.

3) Pretreatment of mammary cancer cells with interferon-gamma enhanced signal transduction and antiproliferative effect of type I interferons (interferon-alpha and interferon-beta), a finding that may lead to improved interferon-based therapy of breast cancer patients.

APPENDIX

Introduction

Breast cancer is the most common malignancy among women in the United States, and the second leading cause of cancer death in that group. In 1998, it is estimated that more than 180,000 women will be diagnosed with invasive carcinoma and that over 40,000 of them will ultimately succumb to the disease (Kopans, 1998). For the individual patient, this translates into a cumulative lifetime risk of 11-12% of developing the disease, and a 3-4% chance of dying (Fischer, et al., 1996; Gail, et al., 1989).

Despite significant improvements in the detection and treatment of the disease, the overall mortality rate has remained essentially unchanged over the past ten years (Fisher, et al., 1996). This is largely due to the increased incidence of the disease, a phenomenon which has been attributed to changing demographics and risk patterns, as outlined below. Recently heightened public awareness about the disease, as well as methods of its detection and prevention will hopefully lead to a decline in overall mortality in the coming decade.

Advances in molecular biology have led to a critical re-evaluation of the previously accepted Halstedian model regarding disease progression. In the past, most physicians believed that breast cancer spread predictably from the primary tumor to the lymph nodes and then to distant sites. Under this model, locoregional disease control was considered curative for cancers that had not yet spread beyond the so-called "sentinel" lymph nodes. It is now known, however, that breast cancer is commonly a systemic disease at the time of first diagnosis (Fisher, et al., 1990). Cancer cells often have been shed during the preclinical phase of tumor growth, and breast cancer can sometimes present as a metastasis without any evidence of a primary tumor. Thus, patients with even very small tumors may have occult micrometastases which can remain dormant for decades or longer (International Breast Cancer Study Group, 1990). While this view of breast cancer as a systemic disease at the time of diagnosis is perhaps less comforting to patients than the Halstedian view, it has allowed for the development of more rational therapies.

With respect to clinical management of breast cancer, the role of the pathologist is perhaps greater than ever. Advances in our understanding of the molecular pathobiology

of the disease now allow for custom-tailored therapy based on the pathologic evaluation of an individual patient's tumor. And, using new objective, molecular-based and highly reproducible staging and grading systems developed by pathologists, clinical researchers are now able to more reliably compare patient groups in their efforts to improve therapy.

Risk factors

A multitude of genetic, hormonal, environmental, and physiologic factors influence a woman's risk of developing breast cancer. Yet 70-80% of women with breast cancer have no apparent risk factors for the disease (Bastarrachea, et al., 1994). These patients are considered to have "sporadic" breast cancer (Gail, et al., 1989).

The most significant risk factor for the development of breast cancer is advancing age (de la Rochefordiere, et al., 1993). The older a woman, the higher her risk of developing breast cancer. This likely relates to the multi-hit theory which states that multiple somatic mutations are required for the development of a malignancy. As the population becomes increasingly older, this risk factor probably contributes to the increasing incidence of breast cancer in the United States.

Hereditary factors are also known to be very important in breast cancer (Biesecker, et al., 1993; Claus, et al., 1994). A woman with a first-degree relative with breast cancer is approximately two to three times more likely to develop the disease than a woman with a negative family history. About 5% of families with breast cancer have a truly hereditary form of the disease (Claus, et al., 1994). First-degree relatives of patients from this group have at least a 50% risk of breast cancer, and some studies suggest that the risk may exceed 90% by age 85 (Biesecker, et al., 1993). A number of factors are used to provide a guide to assessing breast cancer risk for individual women based on familial risk factors. These include the age of the woman at risk and the ages, laterality, and menopausal status of the relatives with breast cancer.

There are several well-defined hereditary syndromes involving breast cancer (Hoskins, et al., 1995). They are potentially important in differential diagnosis and family counseling (Biesecker, et al., 1993). Many patients with Hereditary Breast/Ovarian Cancer Syndrome are believed to have mutant BRCA-1 genes. In one study of families with evidence of linkage to BRCA-1, the lifetime risk of breast cancer was 87% by age 70. The cumulative risk of ovarian

cancer in this study was 44% by age 70 (Berry, et al., 1997). In another study, the group was characterized by breast cancer of no special type, but there was a high frequency of aneuploidy, high S-phase fraction, and patients were younger than normally seen for groups of patients with breast cancer (Miki, et al., 1994).

Hereditary site-specific breast cancer is related to BRCA-2, an autosomal-dominant gene with high penetrance (Easton, et al., 1993), although some of these cases may also be related to other mutant genes yet to be described. Two important clincal characterisitcs of this form of familial breast cancer are early premenopausal onset and bilateral disease, with most of these patients developing breast cancer around age 40. In some families, the presence of the susceptibility allele confers more than a 90% lifetime risk of breast cancer (Hall, et al., 1990). These high-risk individuals are candidates for aggressive breast cancer screening, and possibly even prophylactic surgery.

Li-Fraumeni Syndrome is a rare autosomal-dominant syndrome which predisposes individuals to breast cancer and to a variety of other malignancies (Easton, et al., 1993). These include soft tissue sarcomas, brain tumors, leukemias and lung cancer. The genetic basis of this syndrome probably

resides in inherited mutations in one p53 suppressor allele.

Hormonal regulation of the breast is important in the development of breast cancer (Collaborative Group on Hormonal Factors in Breast Cancer, 1996). Early pregnancy and oophorectomy lower the incidence of the disease, whereas late menopause and early menarche increase the incidence. Lesbians, nuns, and other nulliparous women have a higher risk of breast cancer. Elevated and sustained estrogen levels in such women have been associated with an increased risk of breast cancer (unopposed estrogen effect), but attempts to define and quantify the endocrine factors that may initiate or promote breast cancer have been unsuccessful.

The risk of breast cancer from the use of exogenous hormones is uncertain. Diethylstilbestrol (DES) exposure during pregnancy has been shown to increase the risk of breast cancer, while oral contraceptives do not appear to increase the risk of breast cancer in most women (Collaborative Group on Hormonal Factors in Breast Cancer, 1996). However, meta-analysis of several epidemiologic studies suggests that use of these agents for more than eight years may increase the risk of breast cancer minimally in some subgroups.

Postmenopausal estrogen replacement therapy may affect

the incidence of breast cancer in a dose-related fashion. Some studies have suggested that estrogen, particularly given in high doses, increases the risk of subsequent breast cancer (Collaborative Group on Hormonal Factors in Breast Cancer, 1996). However, several other studies suggest that estrogen given in low doses to relieve menopausal symptoms probably does not increase the incidence of breast cancer.

Benign breast disease may also be a risk factor for subsequent breast cancer. Many studies suggest that lesions with epithelial proliferation or atypia such as hyperplasia, atypical ductal hyperplasia and papillomatosis are prone to malignancy (Del Turco, et al., 1994). And an increased susceptibility to breast cancer has been reported for patients with cyclic mastalgia. It is important to emphasize, however, that the highly common fibrocystic does not increase a patient's risk of developing breast cancer later in life (Fisher, et al., 1996).

Pathological classification

The majority of breast cancers are invasive adenocarcinomas arising from the terminal duct lobular unit. Within this group, most of these tumors are classified as infiltrating ductal carcinomas. Relatively few patients have

the classic form of infiltrating lobular carcinoma, which carries the same prognosis as infiltrating ductal carcinoma. The proportion of patients with in situ carcinomas has been increasing in recent years as a result of the introduction of improved screening techniques such as mammography. Other histologic subtypes of breast cancer are less common, but their identification can be important in planning treatment. The major histological subtypes of breast carcinomas and their incidence asre listed in table 1.

Two major patterns of noninvasive carcinoma are recognized: ductal carcinoma and lobular carcinoma. Ductal carcinoma in situ (DCIS) was considered rare in the past but, with modern mammography, is now much more common. DCIS, as well as its counterpart, lobular carcinoma in situ (LCIS), are premalignant lesions.

DCIS is generally subclassified into two histologic groups: the more common comedo subtype, and the noncomedo form. The majority of comedo DCIS present as movable masses which are well-circumscribed and contain several areas filled with necrotic debris. Microscopically, this lesion is characterized by grossly distended ducts filled with noninvasive cells which may extend into the otherwise disease-free lobules in a process called cancerization. There

Table 1: Mammary Carcinomas by Histological Subtype

Histologic Type	% of Total	
noninvasive	12%	
DCIS		10%
LCIS		2
invasive	88%	
infiltrating ductal carcinoma, NOS		65%
lobular		8
medullary		5
tubular		2
mucinous		2
papillary		1
inflammatory		2
other		3
	100%	100%

From: Fisher, et al., 1996

are large areas of necrosis, with viable cells often found only at the margins. Although noninvasive, these cells may appear highly malignant. Immediate surgical excision is indicated with 5-year survival rates virtually 100%.

The noncomedo form of ductal carcinoma in situ is similar to its counterpart although necrosis is less prominent. The growth rate of these tumors is also generally much lower, consequently they are commonly discovered by mammography. These lesions can vary in size and appearance, and occasionally microinvasion occurs. However, overall prognosis is generally good.

Lobular carcinoma in situ (LCIS) is typically a nonpalpable microscopic lesion discovered by screening mammography. Commonly multicentric or bilateral, LCIS is not only considered a premalignant lesion, but is also associated with a greater risk of developing an invasive breast carcinoma later in life. Thus, although excision is curative, patients with a history of LCIS should be monitored especially closely.

Infiltrating ductal carcinoma is a heterogenous class of tumors which account for approximately 65% of all breast cancers. Compared to the special subtypes (see below), these are generally more aggressive with overall 5-year survival

rates around 60%. In the past, infiltrating ductal carcinomas have typically presented as a hard, palpable masses around 2-3 cm in diameter, occasionally fixed to the underlying fascia or chest wall. Today, many tumors are being detected before they reach 2 cm, an advance of tremendous clinical import.

Macroscopically, infiltrating ductal carcinomas are hard, gritty or chalky masses which commonly assume a stellate shape as they invade into the surrounding stroma. There is tremendous variation microscopically: some tumors may be highly cellular; others involve a profound desmoplastic response. Similarly, there may be wide cytological differences between different tumors. Because of this broad variation, several grading systems have been developed to foster standardization for clinical and research purposes, as discussed below.

Infiltrating lobular carcinoma accounts for approximately 8% of all invasive breast cancers. This lesion is characterized by uniform, small, round, poorly cohesive cells with low-grade nuclear features growing in short, straight, single-file arrangements (Indian files). A targetoid pattern of cells swirling around vessels, ducts and lobules may also be seen. As is the case with its in situ counterpart, infiltrating lobular carcinoma is also more

frequently bilateral. Despite this multicentricity, the overall prognosis for infiltrating lobular carcinoma is good, with 10-year survivals of up to 90% for all stages.

Medullary carcinoma accounts for approximately 5% of all breast cancer. These usually present as a movable, circumscribed mass which upon section appears as a soft, tan and homogenous tumor with occasional areas of necrosis. Microscopically, medullary carcinomas feature a blunt leading edge, polygonal syncytia with high-grade nuclei, a modest stromal component, and often a pronounced lymphocytic infiltrate. Again, these tumors have a prognosis better than that of an infiltrating ductal carcinoma, NOS.

Tubular carcinoma has the best prognosis of the all invasive carcinomas of the breast, with 5-year survival rates approaching 100%. Usually detected mammographically, tubular carcinomas are accompanied by other invasive breast disease in up to 20% of cases. Macroscopically, these lesions appear as small, firm, stellate masses which are composed of small glands or tubules embedded in a dense, fibrous stroma. Histologically, tubular carcinoma may be confused with focal sclerosing adenosis.

Mucinous carcinoma, also called colloid carcinoma, is relatively uncommon and has an excellent prognosis. Usually

presenting as a soft, palpable mass, this cancer will appear as a rubbery, circumscribed tumor upon macroscopic examination. Microscopically, mucinous carcinomas are composed of groups of low-grade malignant cells embedded in a matrix of extracellular material resembling mucin. Although ultimately invasive, the tumor margins may appear circumscribed.

Papillary carcinomas are generally detected as soft, movable, well-circumscribed masses, and are particularly slow-growing. Characterized microscopically by pleomorphic ductal cells with altered polarity, papillary carcinomas are named after the papillae of heaped up cells which grow into the duct lumen. Generally, invasion across the basement membrane occurs very late in the course of the disease. As a consequence, such tumors may be difficult to differentiate from benign papillomatosis.

Inflammatory carcinoma is less a histopathologic diagnosis than a distinct clinical presentation of a previously occult invasive carcinoma. Characterized by pain, redness and swelling of the breast in the area overlying the lesion, inflammatory carcinoma results from the invasion and embolization of the dermal blood vessels and lymphatics. These cancers are often particularly aggressive with a 5-year

survival rate approaching 5%.

A number of sarcomas and lymphomas can arise in the breast. Cystosarcoma phylloides is a rare variant of this group of tumors that has a relatively good prognosis. These tumors are usually large at initial diagnosis, and they grow rapidly. However, despite this large size and rapid growth, axillary lymph node involvement is rare.

Tumor Staging and Grading

It is critical to establish the clinical stage of the disease after a histopathologic diagnosis of breast cancer has been made. Currently, the most widely used clinical staging system is the TNM (tumor-node-metastasis) system of the American Joint Committee on Cancer, depicted in table 2. The establishment of the clinical and pathologic stage of a breast cancer has direct impact on the prognosis and clinical management of the disease (Cascinelli, et al., 1987; Fisher, et al., 1991), as shown in table 3. Although not a reliable, independent prognostic measure alone, the evaluation of tumor grade is of value when placed in the context of tumor stage. While pathologists generally agree on the histological features which characterize high and low grade tumors, there has been no standardized, objective system for grading tumors

Table 2: TNM System for Staging of Breast Cancer

Primary Tumor (T)

TX	Primary tumor cannot be assessed
TO	No evidence of primary tumor
TIS	Carcinoma in Situ: intraductal carcinoma, lobular carcinoma in situ, or Paget's disease of the nipple with no tumor
T1	Tumor <2 cm in greatest dimension
	TIa <0.5 cm in greatest dimension
	TIb >0.5 cm but not >1 cm in greatest dimension
	TIc >1 cm but not >2cm in greatest dimension
T2	Tumor >2 cm but not >5 cm in greatest dimension
T3	Tumor >5 cm in greatest dimension
T4	Tumor of any size with direct extension to chest wall or skin (including ribs, intercostal muscles and serratus anterior muscle, but not pectoral muscle)
	T4a Extension to chest wall
	T4b Edema (including peau d'orange) or ulceration of the skin of the breast or satellite skin nodules confined to the same breast
	T4c Both (T4a and T4b)
	T4d Inflammatory carcinoma

Regional Lymph Node (N)

NX	Regional lymph nodes cannot be assessed (e.g., previously removed)
NO	No regional lymph node metastasis
N1	Metastasis to movable ipsilateral axillary lymph node(s)
N2	Metastasis to ipsilateral axillary lymph node(s) fixed to one another or to other structures
N3	Metastasis to ipsilateral internal mammary lymph node(s)

Pathologic Classification (pN)

pNX	Regional lymph nodes cannot be assessed
pNO	No regional lymph node metastasis
pN1	Metastasis to movable ipsilateral axillary lymph node(s)
pN1a	Only micrometastasis (none >0.2 cm)
pN1b	Metastasis to lymph node(s), any >0.2 cm
pN1bi	Metastasis in 1-3 lymph nodes, any >0.2 cm and all <2 cm
pN1bii	Metastasis to 4+ nodes, any >0.2 cm and all <2 cm
bN1biii	Extension of tumor beyond the capsule of a lymph node metastasis <2 cm
pN1biv	Metastasis to a lymph node >2 cm in greatest dimension
pN2	Metastasis to ipsilateral axillary lymph nodes that are fixed to one another or to other structures
pN3	Metastasis to ipsilateral internal mammary lymph node(s)

Distant Metastasis (M)

MX	Presence of distant metastasis cannot be assessed
MO	No distant metastasis
M1	Distant metastasis (includes metastasis to ipsilateral supraclavicular lymph node(s)

From: Fisher, et al., 1996

Table 3: Survival Statistics by Clinicopathologic Stage

Stage		Survival 5-yr	Survival 10-yr
Stage 0 (in situ)		98%	90%
	Tis, N0, M0		
Stage I			
	T1, N0, M0	95	65
Stage II			
Stage IIA		85	55
	T0, N1, M0		
	T1, N1, M0		
	T2, N0, M0		
Stage IIB		70	45
	T2, N1, M0		
	T3, N0, M0		
Stage III			
Stage IIIA		52	40
	T0, N2, M0		
	T1, N2, M0		
	T2, N2, M0		
	T3, N1, M0		
	T3, N2, M0		
Stage IIIB		50	20
	T4, any N, M0		
	any T, N3, M0		
Stage IV		17	5
	any T, any N, M1		

From: Fisher, et al., 1996

that would allow direct comparison of samples evaluated by different observers.

To overcome this problem several tumor grading systems have been developed (Davis, et al., 1986), the most popular of which is based on evaluation of three quantifiable histological parameters: tubule formation, nuclear pleiomorphism and mitotic rate. Each parameter is given a score from one to three points and the total is used to assign tumor grade: well-, moderately-, or poorly-differentiated.

In addition to the stage and grade of a tumor, several other factors should be considered in assessing prognosis and choosing therapy (Rosen, et al., 1993), as shown in table 4. Tumor characteristics that seem to predict for a higher probability of recurrence include multicentric involvement of the breast (Healey, et al., 1993), the presence of an extensive intraductal component in the breast tumor (as a risk factor for recurrence after segmental mastectomy and radiation therapy), and vascular or lymphatic invasion (as a risk factor for total mastectomy). The number of positive nodes is also critical (Rosen, et al., 1991; Sigurdsson, et al., 1990).

Tumor factors that seem especially important in

Table 4: Clinically Useful Prognostic Factors

Patient:

- age
- menopausal status and age of onset

Clinical features:

- tumor size
- axillary lymph node status

Tumor differentiation:

- histology
- ER/PR status
- karyotype analysis
- ErbB2 overexpression

Metastatic potential:

- demonstrated angiogenesis
- urokinase plasminogen activator
- cathepsin D expression

Proliferative rate:

- S-phase fraction
- mitotic index
- PCNA (proliferating cell nuclear antigen)
- thymidine incorporation rate

From: Fisher, et al., 1996

predicting a poor prognosis from subsequent metastatic disease include the absence of hormone receptors, poorly differentiated histology (especially a high nuclear grade), and tumor invasion of lymphatics, nerves, or venous vascular channels. Studies of the prognostic importance of thymidine incorporation or S-phase fraction as determined by flow cytometry, karyotype analysis have suggested that these are useful as well (Fisher, et al., 1991). An enormous number of other prognostic factors have been identified, many of which are the subject of ongoing trials (Elledge, et al., 1994). Examples include tumor angiogenesis, tumor microvessel density, immunolabeling of PCNA (proliferating cell nuclear antigen), p53 alterations, cathepsin D levels (Tandon, et al., 1990), tissue levels of urokinase and its inhibitor PAI-1, and tumor necrosis in the primary tumor (Gilchrist, et al., 1993).

The evaluation of the estrogen receptor status of a breast cancer has been used for over 20 years to help determine the likely response to hormonal therapy. Estrogen receptor (ER) can be detected in 60-70% of breast cancers, half of which (i.e. a third overall) will respond to hormonal manipulation (Fisher, et al., 1986; Holmes, et al., 1990). The predominant assay has been a ligand-binding method in

which radiolabelled steroid is added to homogenized breast tumor cytosol and binding determined after removal of free steroid by dextran-coated charcoal. This older technique is being supplanted in many laboratories by an enzyme-linked immunosorbent assay. Both approaches allow receptor quantification but require fresh tissue, a limiting factor (Holmes, et al., 1990).

The primary use of ER status determination is its role in the clinical management of breast cancer (Fisher, et al., 1986). Approximately 60% of patient with ER-positive tumors will respond to endocrine therapy, whereas only 10% of those patients with ER-negative tumors will benefit. Unfortunately, a substantial proportion (40%) of ER-positive patients also fail to respond. Several alterations in the estrogen receptor have been identified which could explain this poor clinical outcome (Moot, et al., 1987). It is believed that mutated or truncated forms of the steroid receptor family may have oncogenic potential with aberrant forms competing with normal receptor for binding to hormone response elements and interfering with normal transcription mechanisms. Alterations to the DNA binding domain of the receptor could also be important in disrupting receptor function (Jensen, et al., 1993).

The progesterone receptor (PR) is regulated by estrogen acting through ER and is itself a gene regulator. Along with ER status, it is a useful predictor of response to endocrine therapy (Clark, et al., 1983). PR can be detected by binding assays, similar to those used for ER. Monoclonal antibodies have been developed and these can be used reliably on formalin fixed material, as well as on aspirates. Absence of PR in breast cancers may be due to defects in ER function or to molecular alterations in the PR itself. In general, major gene rearrangements of the PR have not been observed.

The erbB-2 proto-oncogene, also called HER2/neu, has proved to be of particular interest in human breast cancer (Allred, et al., 1992; Paik, et al., 1990). This gene encodes a 185 kD transmembrane glycoprotein, that has extensive homology with epidermal growth factor receptor and is a putative growth factor receptor. Amplification of the gene is found in 20-30% in invasive carcinomas and a correlation has been found between amplification and aggressive features and poor short term prognosis (Gusterson, et al., 1992; Press, et al., 1997). The determination of erbB-2 status is of value in directing therapy since there is clear evidence that erbB-2 positive tumors show a poor response to endocrine therapy (Toikkanen, et al., 1992).

The c-myc gene encodes a nuclear phosphoprotein which acts as a transcriptional regulator, controlling cell proliferation, differentiation and apoptosis. While studies in mice suggest that alterations to this gene may be an important early event in the development of tumors, the evidence from human breast carcinomas is less conclusive (Berns, et al., 1992). Experimentally, alterations to the c-myc gene, predominantly amplification, have been found in approximately 25% of carcinomas and correlate with aggressive features and poor prognosis (Berns, et al., 1992). Clinically, however, c-myc has been of little value in determining prognosis.

Germline p53 mutations have been found in families with the Li-Fraumeni Syndrome, described above. There is an association between the presence of mutations in the p53 gene and aggressive features within breast carcinomas such as negative estrogen receptor status and high S-phase fraction. Some researchers have found a significant association between p53 mutations and disease-free and overall survival (Gasparini, et al., 1994). At present, immunohistochemistry may not always identify mutations, so it is important that there are clearly defined cut-off points for defining positive and negative cases. Besides being of value for the

prediction of prognosis, p53 can aid in the selection of therapy. Generally, adjuvant tamoxifen therapy has been found to be of less value in p53 mutation, lymph node positive cases (Gasparini, et al., 1994). Response to chemotherapy and radiotherapy can also be affected by altered p53 function, due to its role in regulating DNA damage response.

With the increasing emphasis on cancer prevention, many researchers have focused their efforts on the study of useful tumor markers. The term tumor marker refers to any measurable biochemical change in a cell or tissue that is indicative of malignant transformation. That change may take many forms. Perhaps the simplest is a change in the pattern of expression of cell-surface proteins. Many so-called dedifferentiated cancer cells bear proteins ordinarily found only in embryonic tissues; other cancer cells, perhaps as a consequence of loss of enzyme regulation, display improperly processed proteins and carbohydrates; yet others bear mutant proteins.

Because many cellular processes become dysregulated in cancer, malignancy is often marked by dramatic elevations of enzymes that may or may not be normally produced by the given cell type. As is the case with cell-surface markers, these proteins often find their way into the bloodstream and can be detected serologically. Abnormally high levels of circulating

hormones - either made by the neoplastic tissue normally or ectopically - can also be detected in this way. In fact, many cancers are heralded by so-called paraneoplastic syndromes. Growth factors and their receptors may also serve as markers. These may be mutated, or may be present in abnormally large quantities. Steroid receptors, on the other hand, are often down-regulated. A variety of nuclear proteins, among them transcription factors and cell-cycle proteins, are often similarly dysregulated and can be used as tumor markers. Originally applied mainly to hematologic tumors, chromosomal markers, which include often-characterisitc deletions, translocations and amplifications, are now being studied in many solid tumors, as well.

Tumor markers find a variety of applications throughout the clinical course of a cancer. As mentioned above, perhaps the foremost goal of tumor marker research is the development of simple screening tests to detect subclinical disease. Such tests would ideally have high sensitivity and specificity, and would be easily performed on large at-risk populations. More commonly, tumor markers are used in the diagnosis of malignancy as an aid to the staging and grading of a tumor, and can provide insights into the patient's prognosis. Tumor markers are also increasingly used to follow the course of

patients who have already been diagnosed with a cancer. Typically, a baseline marker level will be drawn at the time of diagnosis; the patient will then receive treatment, typically chemotherapy or surgery. Declining levels of the marker are taken to indicate successful intervention. The patient will then be monitored periodically; rising levels of the marker would be taken to indicate a recurrence of the cancer. While straight-forward in principle, marker levels alone are not reliable enough to be used to make clinical judgments.

Among the cell-surface proteins found on malignant mammary epithelial cells are a series of glycoproteins: CEA, TAG-72, CA15-3, CA19-9, muc-1, CD44, and others (Veach, et al., 1987). CEA, a 180kDa glycoprotein which is found not only in breast cancer but lung cancer and colorectal cancer as well, is commonly used to monitor patients post-operatively for recurrence of disease (Moertel, et al., 1993). The protein is sloughed into the bloodstream where it can be assayed; a positive result is judged to be >10ng/ml. Unfortunately, CEA is not specific enough to be useful for screening or diagnostic purposes because a number of benign conditions - eg., liver disease, smoking, fibrocystic disease - cause false positives. Most recently, CEA, along with muc-

1, became the target of a number of immunotherapeutic protocols which enjoyed modest success (McLaughlin, et al., 1996). More specific CEA-like markers include CA15-3 and CA19-9, both of which are under study. TAG-72, another glycoprotein, is the target of a commercially available monoclonal antibody and is used for immunostaining (Bast, et al., 1997).

In addition to the sloughed cell-surface proteins mentioned above, a number of other circulating proteins have been investigated as markers for breast cancer. These include folate binding protein, ferritin, lactate dehydrogenase, lipid-associated sialic acid, urokinase plasminogen activator, cathepsin D, galactosyl transferase, sialyltransferase, fucosyltransferase, casein and lactalbumin (Klee, et al., 1987). Unfortunately, most of these were discovered empirically and are far too nonspecific to be of use in diagnosis or monitoring.

Many clinical pathology laboratories routinely assay breast cancers for several of the growth factor receptors mentioned above, namely EGF and ErbB2 (Press, et al., 1997). The gene product of ErbB2, p185, is a contitutively active growth factor receptor that is overexpressed on the surface of approximately 30% of breast cancers (Paik, et al., 1990).

Presence of this marker is used to judge prognosis; high levels of expression are associated with a short disease-free interval after initial treatment, and reduced overall survival (Muss, et al., 1994). Commercial monoclonal antibodies against p185 are available, and a divalent, cytotoxic, recombinant humanized antibody against p185 developed by Genentech is currently in Phase I clinical trials (Press, et al., 1997).

Several of the nuclear proteins mentioned above also serve as breast cancer markers: p53, p16, Rbl, cyclin Dl, PCNA, and Ki67 are all nonspecific markers that are commonly assayed in breast cancer biopsy samples to aid in tumor grading and prognosis (Wenger, et al., 1993). More recently, the breast cancer-specific BRCA-1 and -2 genes were discovered, but have not come into general use as tumor markers (Wooster, et al., 1994).

Steroid receptors can also be classified as tumor markers in breast cancer. Estrogen receptor and progesterone receptors, as described above, are routinely measured in biopsy samples. Generally, the presence of ER in a tumor is a favorable prognostic sign, whereas ER absence is associated with reduced overall survival. ER negative tumors generally are hormone independent, and do not respond as well to

hormone therapy. Metastatic tumors are more likely than primary tumors to be ER negative. As intimated above, steroid receptor status is widely used to guide treatment: ER positive patients are placed on antiestrogens; ER negative generally are not.

Ectopic hormone production is unknown in breast cancer, and common chromosomal markers are similarly rare, although some cytogenetic techniques are being developed to study commonly amplified genes in breast cancer (Kallioniemi, et al., 1994).

REFERENCES

Ali S, Pellegrini I, Kelly PA: A prolactin-dependent immune cell line (Nb2) expresses a mutant form of prolactin receptor. *J. Biol. Chem.*, 266: 20110-20117, 1991.

Allred DC, Clark GM, Tandon AK: HER-2/neu in node-negative breast cancer: prognostic significance of overexpression influenced by the presence of in situ carcinoma. *J. Clin. Oncol.*, 10: 599-605, 1992.

Archer TK, Zaniewski E, Moyer ML, Nordeen SK: The differential capacity of glucocorticoids and progestins to alter chromatin structure and induce gene expression in human breast cancer cells. *Mol. Endocrinol.*, 8: 1154-62, 1994.

Aylesworth CF, Sylvester PW, Leung FC, Meites J: Inhibition of mammary tumor growth by dexamethasone in rats in the presence of high serum prolactin levels. *Cancer Res.*, 40: 1863-6, 1980.

Bast RC, Mills GB, Gibson S, Boyer CM: Tumor Immunology. In: Cancer Medicine, 4th Ed. Baltimore: Williams and Wilkins, 1997.

Bastarrachea J, Hortobagyi GN, Smith TL, et al.: Obesity as an adverse prognostic factor for patients receiving adjuvant chemotherapy for breast cancer. *Ann. Int. Med.*, 120: 18-25, 1994.

Bazan JF: Structural design and molecular evolution of a cytokine receptor superfamily. *Proc. Natl. Acad. Sci., USA.*, 87: 6934-6938, 1990.

Beadling C, Gushin D, Witthuhn BA, Ziemiecki A, Ihle JN, Kerr IM, Cantrell DA: Activation of Jak kinases and Stat proteins by interleukin-2 and alpha interferon, but not the T-cell antigen receptor, in human T lymphocytes. *EMBO*, 13: 5605-15, 1994.

Berns EM, Klijn JG, van Putten WL, et al.: c-myc amplification is a better prognostic factor than HER2/neu amplification in primary breast cancer. *Cancer Res.*, 52:

1107-1113, 1992.

Bernstein L, Ross RK: Endogenous hormones and breast cancer risk. *Epidemiol. Rev.*, 15: 48-65, 1993.

Berry DA, Parmigiani G, Sanchez J, et al.: Probability of carrying a mutation of breast-ovarian cancer gene BRCA1 based on family history. *J. Natl. Cancer Inst.*, 89: 227-238, 1997.

Biesecker BB, Boehnke M, Calzone K, et al.: Genetic counseling for families with inherited susceptibility to breast and ovarian cancer. *JAMA*, 269: 1970-1974, 1993.

Biswas R, Vonderhaar BK: Role of serum in the prolactin responsiveness of MCF-7 human breast cancer cells in long-term tissue culture. *Cancer Res.*, 47: 3509-3514, 1987.

Black DM, Nicolai H, Borrow J; Solomon E: A somatic cell hybrid map of the long arm of human chromosome 17, containing the familial breast cancer locus (BRCA1). *Am. J. Hum. Genet.*, 52: 702-10, 1993.

Bluyssen HA, Muzaffar R, Vlieststra RJ, van der Made AC, Leung S, Stark GR, Kerr IM, Trapman J, Levy DE: Combinatorial

association and abundance of components of interferon-stimulated gene factor 3 dictate the selectivity of interferon responses. *Proc. Natl. Acad. Sci., USA.*, 92: 5645-9, 1995.

Bogushki MS, McCormick F: Proteins regulating RAS and its relatives. *Nature* 366: 643-653, 1993.

Bolander FF, Nicholas FKR, Van Wyk JJ, Topper YJ: Insulin is essential for accumulation of casein mRNA in mouse mammary epithelial cells. *Proc. Natl. Acad. Sci., USA.*, 78: 5682-4, 1981.

Bonneterre J, Peyrat JP, Beuscart R, Demaille A: Biological and clinical aspects of prolactin receptors in human breast cancer. *J. Steroid Biochem. Mol. Biol.*, 37: 977-981, 1990.

Borellini F, Oka T: Growth control and differentiation in mammary epithelial cells. *Environ. Health Perspect.*, 80: 85-99, 1989.

Boutin JM, Jolicouer C, Okamura H, Gagnon J, Edery M, Shirota M, Banville D, Dusanter-fort I, Djiane J, Kelly PA: Cloning

and expression of the rat prolactin receptor, a member of the growth hormone/prolactin receptor family. *Cell*, 53: 69-77, 1988.

Breast. In: American Joint Committee on Cancer: Manual for Staging of Cancer. Philadelphia: JB Lippincott Company, 4th ed., 1992, pp 149-154.

Bromberg JF, Horvath CM, Wen Z, Schreiber RD, Darnell JEJr: Transcriptionally active Stat1 is required for the antiproliferative effects of both interferon alpha and interferon gamma. *Proc. Natl. Acad. Sci., USA.*, 93: 7673-7678, 1996.

Buck K, Vanek M, Groner B, Ball RK: Multiple forms of prolactin receptor messenger ribonucleic acid are specifically expressed and regulated in murine tissues and the mammary cell line HC11. *Endocrinology*, 130: 1108-1114, 1992.

Buckley AR, Putnam CW, Russell DH: Prolactin is a tumor promoter in rat liver. *Life Sci.*, 37: 2569-2575, 1985.

Buckley AR, Rao YP, Buckley DJ, Gout PW: Prolactin-induced phosphorylation and nuclear translocation of MAP kinase in Nb2 lymphoma cells. *Biochem. Biophys. Res. Commun.*, 204: 1158-64, 1994.

Carey GB, Liberti JP: Stimulation of receptor-associated kinase, tyrosine kinase, and MAP kinase is required for prolactin-mediated macromolecular biosynthesis and mitogenesis in Nb2 lymphoma. *Arch. Biochem. Biophys.*, 316: 179-89, 1995.

Cascinelli N, Greco M, Bufalino R, et al.: Prognosis of breast cancer with axillary node metastases after surgical treatment only. *Eur. J. Cancer Clin. Oncol.*, 23: 795-799, 1987.

Chen HJ, Bradley CJ, Meites J: Stimulation of carcinogen-induced mammary tumor growth in rats by adrenalectomy. *Cancer Res.*, 36: 1414-7, 1976.

Chiba T, Amanuma H, Todokoro K: Tryptophan residue of Trp-Ser-X-Trp-Ser motif in extracellular domains of erythropoietin receptor is essential for signal transduction.

Biochem. Biophys. Res. Commun., 184: 485-90, 1992.

Chin YE, Kitagawa M, Su WS, You ZH, Iwamoto Y, Fu XY: Cell growth arrest and induction of cyclin-dependent kinase inhibitor p21 WAF1/CIP1 mediated by Stat1. *Science*, 272: 719-22, 1996.

Clark GM, McGuire WL, Hubay CA, et al.: Progesterone receptors as a prognostic factor in stage II breast cancer. *NEJM*, 309: 1343-1347, 1983.

Claus EB, Risch N, Thompson WD: Autosomal dominant inheritance of early-onset breast cancer: implications for risk prediction. *Cancer*, 73:643-651, 1994.

Clevenger CV, Chang WP, Ngo W, Pasha TM, Montone KT, Tomaszewski JE: Expression of prolactin and prolactin receptor in human breast carcinoma: Evidence for an autocrine/paracrine loop. *Am. J. Pathol.*, 146: 695-705, 1995.

Clevenger CV, Torigoe T, Reed JC: Prolactin induces rapid phosphorylation and activation of prolactin receptor-associated RAF-1 kinase in a T-cell line. *J. Biol. Chem.*, 269; 5559-5565, 1994.

Collaborative Group on Hormonal Factors in Breast Cancer: Breast cancer and hormonal contraceptives: collaborative reanalysis of individual data on 53,297 women with breast cancer and 100,239 women without breast cancer from 54 epidemiological studies. *Lancet*, 347: 1713-1727, 1996.

Colosi P, Wong K, Leong SR, Wood WI: Mutational analysis of the intracellular domain of the human growth hormone receptor. *J. Biol. Chem.*, 268: 12617-23, 1993.

Coradini D, Biffi A, Pirronello E, Di Fronzo G: The effect of alpha-, beta, and gamma-interferon on the growth of breast cancer cell lines. *Anticancer Res.*, 14: 1779-84, 1994.

Cosman D: The hematopoietin receptor superfamily. *Cytokine*, 5: 95-106, 1993.

D' Cunha J, Knight E, Haas AL, Truitt RL, Borden EC: Immunoregulatory properties of ISG15, an interferon-induced cytokine. *Proc. Natl. Acad. Sci., USA.*, 93:211-215, 1996.

Danial NN, Pernis A, Rothman PB: Jak-STAT signaling induced

by the v-abl oncogene. *Science*, 269: 1875-7, 1995.

Darnell JE: The JAK-STAT pathway: summary of initial studies and recent advances. *Recent Prog. Horm. Res.*, 51: 391-403, 1996.

Darnell JE, Kerr IM, Stark GR: Jak-Stat pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science*, 264: 1415-21, 1994.

Darnell JE: Stats and gene regulation. *Science*, 277: 1630-5, 1997.

Das R, Vonderhaar BK: Involvement of SHC, GRB2, SOS and RAS in prolactin signal transduction in mammary epithelial cells. *Oncogene*, 13: 1139-45, 1996a.

Das R, Vonderhaar BK: Activation of raf-1, MEK, and MAP kinase in prolactin responsive mammary cells. *Breast Cancer Res. Treat.*, 40: 141-9, 1996b.

DaSilva L, Howard OM, Rui H, Kirken RA, Farrar WL: Growth signaling and Jak2 activation mediated by membrane-proximal

cytoplasmic regions of prolactin receptors. *J. Biol. Chem.*, 269:18267-70, 1994.

DaSilva L, Rui H, Erwin RA, Howard OM, Kirken RA, Malabarba MG, Hackett RH, Larner AC, Farrar WL: Prolactin recruits STAT1, STAT3 and STAT5 independent of conserved receptor tyrosines TYR402, TYR479, TYR515 and TYR580. *Mol. Cell. Endocrinol.*, 117: 131-40, 1996.

David M, Petricoin E, Benjamin C, Pine R, Weber MJ, Larner AC: Requirement for MAP kinase (ERK2) activity in interferon alpha- and interferon beta-stimulated gene expression through Stat proteins. *Science*, 269: 1721-3, 1995.

David M, Petricoin EF, Igarashi K, Feldman GM, Finbloom DS, Larner AC: Prolactin activates the interferon-regulated p91 transcription factor and the Jak2 kinase by tyrosine phosphorylation. *Proc. Natl. Acad. Sci., USA.*, 91: 7174-8, 1994.

Davis BW, Gelber RD, Goldhirsch A, et al.: Prognostic significance of tumor grade in clinical trials of adjuvant therapy for breast cancer with axillary lymph node

metastasis. *Cancer*, 58: 2662-2670, 1986.

de la Rochefordiere A, Asselain B, Campana F, et al.: Age as prognostic factor in premenopausal breast carcinoma. *Lancet*, 341:1039-1043, 1993.

Del Turco MR, Palli D, Cariddi A, et al.: Intensive diagnostic follow-up after treatment of primary breast cancer: a randomized trial. *JAMA*, 271: 1593-1597, 1994.

Dickson RB, Lippman ME: Growth factors in breast cancer. *Endocrine Reviews*, 16: 559-89, 1995.

Doppler W, Groner B, Ball RK: Prolactin and glucocorticoid hormones synergistically induce expression of transfected rat beta-casein gene promoter constructs in a mammary epithelial cell line. *Proc. Natl. Acad. Sci., USA.*, 86: 104-8, 1989.

Doppler W, Hock W, Hofer P, Groner B, Ball RK: Prolactin and glucocorticoid hormones control transcription of the beta-casein gene by kinetically distinct mechanisms. *Mol. Endocrinol.*, 4: 912-9, 1990.

Doppler W: Regulation of gene expression by prolactin. *Rev.*

Physiol. Biochem. Pharm., 124: 93-131, 1994.

Eastern Cooperative Oncology Group. Adjuvant systemic therapy in premenopausal (CMF, CMFP, CMFPT) and postmenopausal (observation, CMFP, CMFPT) women with node positive breast cancer. In: S. E. Jones and S. E. Salmon (eds.): Adjuvant Therapy of Cancer IV, pp. 359-368. Orlando: Grune and Stratton, 1984.

Easton DF, Bishop DT, Ford D, et al.: Genetic linkage analysis in familial breast and ovarian cancer: results from 214 families. *Am. J. Human Gen.*, 52: 678-701, 1993.

Einhorn S, Grander D: Why do so many cancer patients fail to respond to interferon therapy? *J. Interferon Cytokine Res.*, 16: 275-281, 1996.

Einhorn S, Strander H: Interferon treatment of human malignancies. *Med. Oncol. Tumor Pharmacother.*, 10: 25-29, 1993.

Elberg G, Kelly PA, Djiane J, Binder L, Gertler A: Mitogenic and binding properties of monoclonal antibodies to the prolactin receptor in Nb2 rat lymphoma cells. Selective

enhancement by anti-mouse IgG. *J. Biol. Chem.*, 265: 14770-14776, 1990.

Elledge RM, Clark GM, Chamness GC, et al.: Tumor biologic factors and breast cancer prognosis among white, Hispanic, and black women in the United States. *J. Natl. Cancer Inst.*, 86: 705-712, 1994.

Engel LW, Young NA: Human breast carcinoma cells in continuous culture: a review. *Cancer Res.*, 38: 4327-39, 1978.

Erwin RA, Kirken RA, Malabarba MG, Farrar WL, Rui H: Prolactin activates Ras via signaling proteins SHC, growth factor receptor bound 2, and son of sevenless. *Endocrinology*, 136: 3512-8, 1995.

Fanager H, Ree HJ: Cyclic changes of human mammary gland epithelium in relation to the menstrual cycle. *Cancer*, 34: 574-85, 1974.

Fentiman IS, Howell A, Hamed H, Lee SM, Ranson M, Wall J, Chaudary MA, Ash CM, Gregory Wm, Sellwood RA: A controlled trial of adjuvant tamoxifen, with or without prednisolone, in

post-menopausal women with operable breast cancer. *Br. J. Cancer*, 70: 729-31, 1994.

Fields K, Kulig E, Lloyd RV: Detection of prolactin messenger RNA in mammary and other normal and neoplastic tissues by polymerase chain reaction. *Lab. Invest.*, 68: 354-360, 1993.

Finbloom DS, Larner AC: Regulation of the Jak-Stat signaling pathway. *Cell Signal*, 7: 739-45, 1995.

Fisher B, Fisher ER, Redmond C, et al.: Tumor nuclear grade, estrogen receptor, and progesterone receptor: their value alone or in combination as indicators of outcome following adjuvant therapy for breast cancer. *Breast Cancer Res. Treat.*, 7: 147-160, 1986.

Fisher B, Gunduz N, Constantino J, et al.: DNA flow cytometric analysis of primary operable breast cancer: relation of ploidy and s-phase fraction to outcome of patients in NSABP B-04. *Cancer*, 68: 1465-1475, 1991.

Fisher B, Osborne CK, Margolese R, et al.: Neoplasms of the breast. In: Holland JF, Frei E, Bast RC, et al., Eds.: Cancer

Medicine. Philadelphia: Lea & Febiger, 3rd ed., 1996, pp 1706-1716.

Fisher ER, Redmond C, Fisher B, et al.: Pathologic findings from the National Surgical Adjuvant Breast and Bowel Projects (NSABP): prognostic discriminants for 8-year survival for node-negative invasive breast cancer patients. *Cancer*, 65(Suppl): 2121-2128, 1990.

Fleischmann WR, Newton RC, Fleischmann CM, Colburn NH, Brysk MM: Discrimination between nonmalignant and malignant cells by combination of IFN-gamma and IFN-alpha/beta. *J. Biol. Response Mod.*, 3: 397-405, 1984.

Foxwell BM, Barrett K, Feldmann M: Cytokine receptors: structure and signal transduction. *Clin. Exp. Immunol.* 90: 161-169, 1992.

Freshney RI: Culture of Animal Cells: A Manual of Basic Technique, 3d Ed., New York: Wiley-Liss, 1994.

Friedman RM: Antitumor Effects of Interferons. *J. Exp. Path.*, 3: 203-222, 1987.

Fuh G, Wells JA: Prolactin receptor antagonists that inhibit the growth of breast cancer cell lines. *J. Biol. Chem.*, 270: 13133-7, 1995.

Futreal PA, Soderkvist P, Marks JR, Iglehart JD, Cochran C, Barrett JC, Wiseman RW: Detection of frequent allelic loss on proximal chromosome 17q in sporadic breast carcinoma using microsatellite length polymorphisms. *Cancer Res.*, 52: 2624-7, 1992.

Gail MH, Brinton LA, Byar DP, et al.: Projecting individualized probabilities of developing breast cancer for white females who are being examined annually. *J. Natl. Cancer Inst.*, 81: 1879-1886, 1989.

Garcia R, Yu CL, Hudnall A, Catlett R, Nelson KL, Smithgall T, Fujita DJ, Ethier SP, Jove R: Constitutive activation of Stat3 in fibroblasts transformed by diverse oncoproteins and in breast carcinoma cells. *Cell. Growth Differ.*, 8: 1267-76, 1997.

Gasparini G, Weidner N, Bevilacqua P, et al.: Tumor

microvessel density, p53 expression, tumor size, and peritumoral lymphatic vessel invasion are relevant prognostic markers in node-negative breast carcinoma. *J. Clin. Oncol.*, 12: 454-466, 1994.

Ghislain JJ, Fish EN: Application of genomic DNA affinity chromatography identifies multiple interferon-alpha-regulated Stat2 complexes. *J. Biol. Chem.*, 271: 12408-13, 1996.

Gilchrist KW, Gray R, Fowble B, et al.: Tumor necrosis is a prognostic predictor for early recurrence and death in lymph node-positive breast cancer: a 10-year follow-up study of 728 Eastern Cooperative Oncology Group patients. *J. Clin. Oncol.*, 11: 1929-1935, 1993.

Ginsburg E, Vonderhaar BK: Prolactin Synthesis and Secretion by Human Breast Cancer Cells. *Cancer Res.*, 55: 2591-2595, 1995.

GIVIO Investigators: Impact of follow-up testing on survival and health-related quality of life in breast cancer patients: a multicenter randomized controlled trial. *JAMA*, 271: 1587-1592, 1994.

Gouilleux F, Wakao H, Mundt M, Groner B: Prolactin induces phosphorylation of Tyr694 of Stat5 (MGF), a prerequisite for DNA binding and induction of transcription. *EMBO*, 13: 4361-9, 1994.

Groner B, Gouilleux F: Prolactin-mediated gene activation in mammary epithelial cells. *Curr. Opin. Genet. Dev.*, 5: 587-94, 1995.

Gusterson BA, Gelber RD, Goldhirsch A, et al.: Prognostic importance of c-erbB-2 expression in breast cancer. *J. Clin. Oncol.*, 10: 1049-1056, 1992.

Hall JM, Lee MK, Newman B, et al.: Linkage of early-onset familial breast cancer to chromosome 17q21. *Science*, 250: 1684-1689, 1990.

Han Y, Watling D, Rogers NC, Stark GR: JAK2 and STAT5, but not JAK1 and STAT1, are required for prolactin-induced beta-lactoglobulin transcription. *Mol. Endocrinol.*, 11: 1180-8, 1997.

Hansen RM, Borden EC: Current status of interferons in the treatment of cancer. *Oncology*, 11: 19-24, 1992.

Happ B, Groner B: The activated mammary gland specific nuclear factor (MGF) enhances in vitro transcription of the beta-casein gene promoter. *J. Steroid Biochem. Mol. Biol.*, 47: 21-30, 1993.

Harlow E, Lane DP: Antibodies: A Laboratory Manual, Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1988.

Healey EA, Cook EF, Orav EJ, et al.: Contralateral breast cancer: clinical characteristics and impact on prognosis. *J. Clin. Oncol.*, 11: 1545-1552, 1993.

Holmes FA, Fritsche HA, Loewy JW, et al.: Measurement of estrogen and progesterone receptors in human breast tumors: enzyme immunoassay versus binding assay. *J. Clin. Oncol.*, 8: 1025-1035, 1990.

Hoskins KF, Stopfer JE, Calzone KA, et al.: Assessment and counseling for women with a family history of breast cancer: a guide for clinicians. *JAMA*, 273: 577-585, 1995.

Hundertmark S, Buhler H, Rudolf M, Weitzel HK, Ragosch V: Inhibition of 11 beta-hydroxysteroid dehydrogenase activity enhances the antiproliferative effect of glucocorticosteroids on MCF-7 and ZR-75-1 breast cancer cells. *J. Endocrinol.*, 155: 171-80, 1997.

Ihle JN, Kerr IM: Jaks and Stats in signaling by the cytokine receptor superfamily. *Trends Genet.*, 11: 69-74, 1995a.

Ihle JN, Witthuhn BA, Quelle FW, Yamamoto K, Silvennoinen O: Signaling through the hematopoietic cytokine receptors. *Ann. Rev. Immunol.*, 13: 369-98, 1995b.

Ihle JN: Stats and MAPKs: obligate or opportunistic partners in signaling. *Bioessays*, 18: 95-8, 1996.

Ingle JN, Mailliard JA, Schaid DJ, Krock JE, Gesme DH, Windschitl HE, Pfeifle DM, Etzell PS, Gerstner JG, Long HJ: A double-blind trial of tamoxifen plus prednisolone versus tamoxifen plus placebo in postmenopausal women with metastatic breast cancer. A collaborative trial of the North Central Cancer Treatment Group and Mayo Clinic. *Cancer*, 68:

34-9, 1991.

Ingram DM, Nottage EM, Roberts AN: Prolactin and breast cancer risk. *Med. J. Aust.*, 153: 469-470, 1991.

International (Ludwig) Breast Cancer Study Group: Prognostic importance of occult axillary lymph node micrometastases from breast cancers. *Lancet*, 335: 1565-1568, 1990.

Jensen EV, DeSombre ER: Steroid hormone binding and hormone receptors. In: Holland JF, Frei E, Bast RC, et al., Eds.: Cancer Medicine. Philadelphia: Lea and Febiger, 3rd ed., 1993, pp 815-823.

Juergens WG, Stockdale FE, Topper YJ, Elias JJ: Hormone-dependent differentiation of mammary gland in vitro. *Proc. Natl. Acad. Sci. USA*, 54: 629-34, 1965.

Kalache A, Maguire A, Thompson SG: Age at last full-term pregnancy and risk of breast cancer. *Lancet*, 341: 33-36, 1993.

Kallioniemi A: Development of molecular and cytogenetic techniques for analysis of DNA amplification in breast

cancer. *Acta Universitatis Tamperensis*, 419:1-214, 1994.

Kazlauskas A, Feng GS, Pawson T, Valius M: The 64-kilodalton protein that associates with the platelet-derived growth factor beta subunit via Tyr-1009 is the SH2-containing phosphotyrosine phosphatase SYP. *Proc. Natl. Acad. Sci., USA.*, 90: 6939-6943, 1993.

Kelly PA, Ah S, Rozakis M, Goujon L, Nagano G, Pellegrini I, Gould D, Djiane J, Edery M, Finidori J, Postel-Vinay MC: The growth hormone/prolactin receptor family. *Rec. Prog. Hor. Res.*, 48: 123-163, 1993.

Kinzler KW, Vogelstein B: Lessons from hereditary colorectal cancer. *Cell*, 87: 159-70, 1996.

Kirken RA, Malabarba MG, Xu J, Liu X, Farrar WL, Hennighausen L, Larner AC, Grimley PM, Rui H: Prolactin stimulates serine/tyrosine phosphorylation and formation of heterocomplexes of multiple Stat5 isoforms in Nb2 lymphocytes. *J. Biol. Chem.*, 272: 14098-103, 1997a.

Kirken RA, Malabarba MG, Xu J, DaSilva L, Erwin RA, Liu X, Hennighausen L, Rui H, Farrar WL: Two discrete regions of

interleukin-2 (IL2) receptor beta independently mediate IL2 activation of a PD98059/rapamycin/wortmannin-insensitive Stat5a/b serine kinase. *J. Biol. Chem.*, 272: 15459-65, 1997b.

Kirken RA, Rui H, Evans GA, Farrar WL: Characterization of an interleukin-2 (IL-2)-induced tyrosine phosphorylated 116-kDa protein associated with the IL-2 receptor beta-subunit. *J. Biol. Chem.*, 268: 22765-22770, 1993.

Klee GG, Go VLW: Carcinoembryonic antigen and its role in clinical practice. In: Ghosh BC, Ghosh L, Eds.: Tumor Markers and Tumor-Associated Antigens, New York: McGraw Hill, 1987.

Klein G: Multistep emancipation of tumors from growth control: can it be curbed in a single step? *Bioessays*, 12: 347-350, 1990.

Kopans DB: Breast Imaging, 2d Ed., Philadelphia: Lippincott-Raven, 1998.

Labrie F, Poulin R, Simard J, Zhao HF, Labrie C, Dauvois S, Dumont M, Hatton AC, Poirier D, Merand Y: Interactions between estrogens, androgens, progestins, and glucocorticoids

in ZR-75-1 human breast cancer cells. *Ann. N. Y. Acad. Sci.*, 595: 130-48, 1990.

Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 277: 680-688, 1970.

Lebrun JJ, Ali S, Sofer L, Ullrich A, Kelly PA: Prolactin-induced proliferation of Nb2 cells involves tyrosine phosphorylation of the prolactin receptor and its associated tyrosine kinase JAK2. *J. Biol. Chem.*, 269: 14021-14026, 1994.

Lechner J, Welte T, Tomasi JK, Bruno P, Cairns C, Gustafsson J, Doppler W: Promoter-dependent synergy between glucocorticoid receptor and Stat5 in the activation of beta-casein gene transcription. *J. Biol. Chem.*, 272: 20954-60, 1997.

Li X, Leung S, Qureshi S, Darnell JE, Stark GR: Formation of Stat1-Stat2 heterodimers and their role in the activation of IRF-1 gene transcription by alpha-interferon. *J. Biol. Chem.*, 271: 5790-4, 1996.

Lin JX, Mietz J, Modi WS, John S, Leonard WJ: Cloning of human Stat5B. Reconstitution of interleukin-2-induced Stat5A and Stat5B DNA binding activity in COS-7 cells. *J. Biol. Chem.*, 271: 10738-44, 1996.

Liu X, Robinson GW, Gouilleux F, Groner B, Hennighausen L: Cloning and expression of Stat5 and an additional homologue (Stat5b) involved in prolactin signal transduction in mouse mammary tissue. *Proc. Natl. Acad. Sci., USA.*, 92: 8831-5, 1995.

Liu X, Robinson GW, Wagner KU, Garrett L, Wynshaw-Boris A, Hennighausen L: Stat5a is mandatory for adult mammary gland development and lactogenesis. *Genes Dev.*, 11: 179-86, 1997.

Longacre TA, Bartow SA: A correlative morphological study of human breast and endometrium in the menstrual cycle. *Am. J. Surg. Path.*, 10: 382-93, 1986.

Love RR, Rose DR, Surawicz TS, Newcomb PA: Prolactin and growth hormone levels in premenopausal women with breast cancer and healthy women with a strong family history of breast cancer. *Cancer*, 68: 1401-5, 1991.

Macheledt JE, Buzdar AU, Hortobagyi GN, Frye DK, Gutterman JU, Holmes FA: Phase II evaluation of interferon added to tamoxifen in the treatment of metastatic breast cancer. *Breast Cancer Res. Treat.*, 18: 165-70, 1991.

Maddox PR, Jones DL, Mansel RE: Prolactin and total lactogenic hormone measured by microbioassay and immunoassay in breast cancer. *Br. J. Cancer*, 65: 456-460, 1992.

Malarkey WB, Kennedy M, Allred LE, Milo G: Physiological concentrations of prolactin can promote the growth of human breast tumor cells in culture. *J. Clin. Endocrinol. Metab.*, 56: 673-677, 1983.

Manni A, Wright C, Davis G, Glenn J, Joehl R, Feil P: Promotion by prolactin of the growth of human breast neoplasms cultured in vitro in the soft agar clonogenic assay. *Cancer Res.*, 46: 1669-1672, 1986.

Manni A: Endocrine therapy of breast and prostate cancer. *Endocrinol. Metab. Clin. North Am.*, 18: 569-92, 1989.

Marengere LE, Songyang Z, Gish GD, Schaller MD, Parsons JT,

Stern MJ, Cantley LC, Pawson T: SH2 domain specificity and activity modified by a single residue. *Nature*, 369: 502-505, 1994.

McLaughlin JP, Schlom L, Kantor JA, Greiner JW: Improved immunotherapy of a recombinant carcinoembryonic antigen vaccinia vaccine when given in combination with interleukin 2. *Cancer Res.*, 56: 2361-2367, 1996.

McLeod GR, Thomson DB, Hersey P: Clinical evaluation of interferons in malignant melanoma. *J. Invest. Dermatol.*, 185-187, 1990.

Meakin JW, Allt WE, Beale FA, Bush RS, Clark RM, Fitzpatrick PJ, Hawkins NV, Jenkin RD, Pringle JF, Reid JG: Ovarian irradiation and prednisone following surgery and radiotherapy for carcinoma of the breast. *Breast Cancer Res. Treat.*, 3: 45-8, 1983.

Meinke A, Barahmand-Pour F, Wohrl S, Stoiber D, Decker T: Activation of different Stat5 isoforms contributes to cell-type-restricted signaling in response to interferons. *Mol. Cell. Biol.*, 16: 6937-44, 1996.

Merlo GR, Graus-Porta D, Cella N, Marte BM, Taverna D, Hynes NE: Growth, differentiation and survival of HC11 mammary epithelial cells: diverse effects of receptor tyrosine kinase-activating peptide growth factors. *Eur. J. Cell Biol.*, 70: 97-105, 1996.

Mershon J, Sall W, Mitchner N, Ben-Jonathan N: Prolactin is a local growth factor in rat mammary tumors. *Endocrinology*, 136: 3619-23, 1995.

Meydan N, Grunberger T, Dadi H, Shahar M, Arpaia E, Lapidot Z, Leeder JS, Freedman M, Cohen A, Gazit A, Levitzki A, Roifman CM: Inhibition of acute lymphoblastic leukaemia by a Jak-2 inhibitor. *Nature*, 379: 645-8, 1996.

Meyer J, Juecker M, Ostertag W, Stocking C: Carboxyl-truncated STAT5-beta is generated by a nucleus-associated serine protease in early hematopoietic progenitors. *Blood*, 91: 1901-8, 1998.

Migone TS, Lin JX, Cereseto A, Mulloy JC, O'Shea JJ, Franchini G, Leonard WJ: Constitutively activated Jak-STAT

pathway in T cells transformed with HTLV-I. *Science*, 269: 79-81, 1995.

Miki Y, Swensen J, Shattuck-Eidens D, et al.: A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science*, 266: 66-71, 1994.

Miller WR, Shivas AA, Franchimont P, Haagensen DE: Breast gross cystic disease protein 15 in human breast cancer in culture. *Eur. J. Cancer Clin. Oncol.*, 24: 223-8, 1988.

Minami Y, Oishi I, Liu ZJ, Nakagawa S, Miyazaki T, Taniguchi T: Signal transduction mediated by the reconstituted IL-2 receptor. Evidence for a cell type-specific function of IL-2 receptor beta chain. *J. Immunol.*, 152: 5680-5690, 1994.

Miyajima A, Hara T, Kitamura T: Common subunits of cytokine receptors and the functional redundancy of cytokines. *TIBS*. 17: 378-382, 1990.

Moertel CG, Fleming TR, McDonald JS, Haller DG, Laurie JA, Tangen C: An evaluation of the carcinoembryonic antigen (CEA) test for monitoring patients with resected colon cancer.

JAMA, 270: 943-947, 1993.

Moodie SA, Willumsen BM, Weber MJ, Wolfman A: Complexes of RAS-GTP with RAF-1, and mitogen-activated protein kinase kinase. *Science,* 260: 1658-1660, 1993.

Moot SK, Peters GN, Cheek JH: Tumor hormone receptor status and recurrences in premenopausal node negative breast carcinoma. *Cancer,* 60: 382-385, 1987.

Murphy LJ, Murphy LC, Vrhovsek E, Sutherland RL, Lazarus L: Correlation of lactogenic receptor concentration in human breast cancer with estrogen receptor concentration. *Cancer Res.*, 44: 1963-8, 1984.

Muss HB, Thor AD, Berry DA, et al.: c-erbB-2 expression and response to adjuvant therapy in women with node-positive early breast cancer. *NEJM,* 330: 1260-1266, 1994.

Myal Y, Robinson DB, Iwasiow B, Tsuyuki D, Wong P, Shiu RP: The prolactin-inducible protein (PIP/GCDFP-15) gene: cloning, structure and regulation. *Mol. Cell. Endocrinol.*, 80: 165-75, 1991.

Nakamura A, Shirai T, Ogawa K, Wada S, Fujmoto NA, Ito N: Promoting action of prolactin released from a grafted transplantable pituitary tumor (MtT/F84) on rat prostate carcinogenesis. *Cancer Lett.*, 53: 151-157, 1990.

Nandi S, Guzman RC, Yang J: Hormones and mammary carcinogenesis in mice, rats and humans: a unifying hypothesis. *Proc. Natl. Acad. Sci., USA.*, 92: 3650-7, 1995.

O'Neal KD, Montgomery DW, Truong TM, Yu-Lee LY: Prolactin gene expression in human thymocytes. *Mol. Cell. Endocrinol.*, 87: 19-23, 1992.

O' Shea JJ: Jaks, Stats, cytokine signal transduction, and immunoregulation: Are we there yet? *Immunity*, 7: 1-11, 1997.

Offit K, Gilewski T, McGuire P, et al.: Germline BRCA1 185delAG mutations in Jewish women with breast cancer. *Lancet*, 347:1643-1645, 1996.

Ouhtit A, Morel G, Kelly PA: Visualisation of gene expression of short and long forms of prolactin receptor in the rat.

Endocrinology, 133: 135-144, 1993.

Paik S, Hazan R, Fisher ER, et al.: Pathologic findings from the National Surgical Adjuvant Breast and Bowel Project: prognostic significance of erbB-2 protein overexpression in primary breast cancer. *J. Clin. Oncol.*, 8: 103-112, 1990.

Patthy L: Homology of a domain of the growth hormone/prolactin receptor family with type III modules of fibronectin. *Cell*, 61: 13-14, 1990.

Pawson T: Protein-tyrosine kinases: Getting down to specifics. *Nature*, 373: 477-478, 1995.

Pawson T: Regulation of the RAS signalling pathway by protein-tyrosine kinases. *Biochem. Soc. Trans.*, 22: 455-460, 1994.

Peeters P, Raynaud SD, Cools J, Wlodarska I, Grosgeorge J, Philip P, Monpoux F, Van Rompaey L, Baens M, Van den Berghe H, Marynen P: Fusion of TEL, the ETS-variant gene 6 (ETV6), to the receptor-associated kinase JAK2 as a result of t(9; 12) in a lymphoid and t(9; 15; 12) in a myeloid leukemia. *Blood*, 90: 2535-40, 1997.

Peyrat JP, Venin PH, Bonneterre J, Hecquet B, Vandewalle B, Kelly P, Djiane J: Effect of bromocriptine treatment on prolactin and steroid receptor levels in human breast cancer. *Eur. J. Cancer Clin. Oncol.*, 20: 1363-7, 1984.

Press MF, Bernstein L, Thomas PA, et al.: HER-2/neu gene amplification characterized by fluorescence in situ hybridization: poor prognosis in node-negative breast carcinomas. *J. Clin. Oncol.*, 15: 2894-2904, 1997.

Quelle DE, Quelle FW, Wojchowski DM: Mutations in the WSAWSE and cytosolic domains of the erythropoietin receptor affect signal transduction and ligand binding and internalization. *Mol. Cell. Biol.*, 12: 4553-61, 1992.

Ram PA, Park SH, Choi HK, Waxman DJ: Growth hormone activation of Stat 1, Stat 3, and Stat 5 in rat liver. Differential kinetics of hormone desensitization and growth hormone stimulation of both tyrosine phosphorylation and serine/threonine phosphorylation. *J. Biol. Chem.*, 271: 5929-40, 1996.

Rana A, Habib FK, Halliday P, Ross M, Wild R, Elton RA, Chilshon GD: A case for synchronous reduction of testicular androgen, adrenal androgen and prolactin for the treatment of advanced carcinoma of the prostate. *Eur. J. Cancer*, 31: 871-5, 1995.

Reyniak JV: Endocrine physiology of the breast. *J. Reprod. Med.*, 22: 303-9, 1979.

Rosen JM, Humphreys R, Krnacik S, Juo P, Raught B: The regulation of mammary gland development by hormones, growth factors, and oncogenes. *Prog. Clin. Biol. Res.*, 387: 95-111, 1994.

Rosen PP, Groshen S, Kinne DW, et al.: Factors influencing prognosis in node-negative breast carcinoma: analysis of 767 T1N0M0/T2N0M0 patients with long-term follow-up. *J. Clin. Oncol.*, 11: 2090-2100, 1993.

Rosen PP, Groshen S, Kinne DW.: Prognosis in T2N0M0 stage I breast carcinoma: a 20-year follow-up study. *J. Clin. Oncol.*, 9: 1650-1661, 1991.

Roskelley CD, Desprez PY, Bissell MJ: Extracellular matrix-dependent tissue-specific gene expression in mammary epithelial cells requires both physical and biochemical signal transduction. *Proc. Natl. Acad. Sci., USA.*, 91: 12378-82, 1994.

Rubens RD; Tinson CL; Coleman RE; Knight RK; Tong D; Winter PJ; North WR. Prednisolone improves the response to primary endocrine treatment for advanced breast cancer. *Br. J. Cancer*, 58: 626-30, 1988.

Rui H, Djeu JY, Evans GA, Kelly PA, Farrar WL: Prolactin receptor triggering. Evidence for rapid tyrosine kinase activation. *J. Biol. Chem.*, 267: 24076-24081, 1992.

Rui H, Kirken RA, Farrar WL: Activation of receptor-associated tyrosine kinase JAK2 by prolactin. *J. Biol. Chem.*, 269: 5364-8, 1994.

Rui H, Kirken RA, Malabarba MG, Erwin RA, Schaber JD, Duhe RJ, Larner AC, Farrar WL: Cell-dependent activation of tyrosine kinases Jak1, Jak2 and TYK2 by prolactin in human breast cancer and hematopoietic cell lines. Submitted for

publication.

Rui H, Xu J, Dong F, Mehta S, Fang H, Williams J, Grimley PM: Activation of the Jak2-Stat5 signaling pathway in Nb2 lymphoma cells by an anti-apoptotic, aurintricarboxylic acid. *J. Biol. Chem.*, 273: 28-32, 1998.

Sambrook S, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual, Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1989.

Schaber JD, Fang H, Xu J, Grimley PM, Rui H: Prolactin Activates Stat1 but does not Antagonize Stat1-mediated Growth Inhibition by Type I Interferons in Human Breast Cancer Cells. *Cancer Res.*, 58: 1914-19, 1998.

Schindler C, Darnell JE: Transcriptional responses to polypeptide ligands: the Jak-Stat pathway. *Ann. Rev. Biochem.*, 64: 621-51, 1995.

Schlessinger J, Ullrich A: Growth factor signalling by receptor tyrosine kinases. *Neuron*, 9: 383-391, 1992.

Schlessinger J: SH2/SH3 signalling proteins. *Curr. Opin. Gen.*

Dev., 4: 25-30, 1994.

Schmitt-Ney M, Doppler W, Ball RK, Groner B: Beta-casein gene promoter activity is regulated by the hormone-mediated relief of transcriptional repression and a mammary-gland-specific nuclear factor. *Mol. Cell. Biol.*, 11: 3745-55, 1991.

Seymour L, Bezwoda WR: Interferon plus tamoxifen treatment for advanced breast cancer: in vivo biological effects of two growth modulators. *Brit. J. Cancer*, 68: 352-356, 1993.

Shiu RP, Iwasiow BM: Prolactin-inducible proteins in human breast cancer cells. *J. Biol. Chem.*, 260: 11307-11313, 1985.

Shiu RP, Paterson JA: Alteration of cell shape, adhesion, and lipid accumulation in human breast cancer cells (T-47D) by human prolactin and growth hormone. *Cancer Res.*, 44: 1178-1186, 1984.

Shiu RP: Prolactin receptors in human breast cancer cells in long-term tissue culture. *Cancer Res.*, 39: 4381-6, 1979.

Sigurdsson H, Baldetorp B, Borg A, et al.: Indicators of prognosis in node-negative breast cancer. *NEJM*, 322: 1045-

1053, 1990.

Somers W, Ultsch M, De Vos AM, Kossiakoff AA: The X-ray structure of a growth hormone-prolactin receptor complex. *Nature*, 372: 478-81, 1994.

Songyang Z, Shoelson SE, McGlade J, Olivier P, Pawson T, Bustelo XR, Barbacid N, Sabe H, Hanafusa H, Yi T, Ren R, Baltimore D, Ratnofsky S, Feldman RA, Cantley LC: Specific motifs recognized by the SH2 domains of CSK, 3BP2, FES/FPS, GRB2, HCP, SHC, SYK, and VAV. *Mol. Cell. Biol.*, 14: 2777-2785, 1994.

Stahl N, Yancopoulos GD: The alphas, betas, and kinases of cytokine receptor complexes. *Cell*, 74: 587-590, 1993.

Stancato LF, Sakatsume M, David M, Dent P, Dong F, Petricoin EF, Krolewski JJ, Silvennoinen O, Saharinen P, Pierce J, Marshall CJ, Sturgill T, Finbloom DS, Larner AC: Beta interferon and oncostatin M activate Raf-1 and mitogen-activated protein kinase through a Jak1-dependent pathway. *Mol. Cell. Biol.*, 17:3833-40, 1997.

Stewart JF, Rubens RD, King RJ, Minton MJ, Steiner R, Tong D,

Winter PJ, Knight RK, Hayward JL: Contribution of prednisolone to the primary endocrine treatment of advanced breast cancer. *Eur. J. Cancer Clin. Oncol.*, 18: 1307-14, 1982.

Stocklin E, Wissler M, Gouilleux F, Groner B: Functional interactions between Stat5 and the glucocorticoid receptor. *Nature*, 383: 726-8, 1996.

Stoecklin E, Wissler M, Moriggl R, Groner B: Specific DNA binding of Stat5, but not of glucocorticoid receptor, is required for their functional cooperation in the regulation of gene transcription. *Mol. Cell. Biol.*, 17: 6708-16, 1997.

Tanaka M, Maeda K, Okubo T, Nakashima K: Double antenna structure of chicken prolactin receptor deduced from the cDNA sequence. *Biochem. Biophys. Res. Commun.*, 188: 490-6, 1992.

Tandon AK, Clark GM, Chamness GC, et al.: Cathepsin D and prognosis in breast cancer. *NEJM*, 322: 297-302, 1990.

Tejwani GA, Gudehithlu KP, Hanissian SH, Gienapp IE, Whitacre CC, Malarkey WB: Facilitation of dimethylbenz[a] anthracene-

induced rat mammary tumorigenesis by restraint stress: role of beta-endorphin, prolactin and naltrexone. *Carcinogenesis*, 12: 637-641, 1991.

Toikkanen S, Helin H, Isola J, et al.: Prognostic significance of HER-2 oncoprotein expression in breast cancer: a 30-year follow-up. *J. Clin. Oncol.*, 10: 1044-1048, 1992.

Topper YJ, Freeman CS: Multiple hormone interactions in the developmental biology of the mammary gland. *Physiol. Rev.*, 60: 1049-106, 1980.

Udy GB, Towers RP, Snell RG, Wilkins RJ, Park SH, Ram RP, Waxman DJ, Davey HW: Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression. *Proc. Natl. Acad. Sci., USA.*, 94: 7239-44, 1997.

Ullrich A, Schlessinger J: Signal transduction by receptors with tyrosine kinase activity. *Cell*, 61: 203-212, 1990.

Valius M, Bazenet C, Kazlauskas A: Tyrosines 1021 and 1009 are phosphorylation sites in the carboxy terminus of the

platelet-derived growth factor receptor beta subunit and are required for binding of phospholipase C gamma and a 64-kilodalton protein, respectively. *Mol. Cell. Biol.*, 13: 133-143, 1993.

van der Pompe G, Antoni MH, Heijnen CJ: Elevated levels and attenuated ACTH and cortisol responses to behavioral challenge in women with metastatic breast cancer. *Psychoneuroendocrinology*, 21: 361-374, 1996.

Veach SR, Ghosh BC: Biomarkers in breast cancer. In: Ghosh BC, Ghosh L, Eds.: Tumor Markers and Tumor-Associated Antigens. New York: McGraw Hill, 1987.

Vigon I, Mornon JP, Cocault L, Mitjavila MT, Tambourin P, Gisselbrecht S, Souyri M: Molecular cloning and characterization of MPL, the human homolog of the v-mpl oncogene: identification of a member of the hematopoietic growth factor receptor superfamily. *Proc. Natl. Acad. Sci., USA.*, 89: 5640-4, 1992.

Wakao H, Gouilleux F, Groner B: Mammary gland factor (MGF) is a novel member of the cytokine regulated transcription factor

gene family and confers the prolactin response. *EMBO*, 13: 2182-2191, 1994.

Wang DY, De Stavola BL, Bulbrook RD, Allen DS, Kwa HG, Fentiman IS, Hayward JL, Millis RR: Relationship of blood prolactin levels and the risk of subsequent breast cancer. *Int. J. Epidemiol.*, 21: 214-221, 1992.

Wang YF, O'Neal KD, Yu-Lee LY: Multiple prolactin (PRL) receptor cytoplasmic residues and Stat1 mediate PRL signaling to the Interferon Regulatory Factor-1 promoter. *Mol. Endocrin.*, 11: 1353-1364, 1997.

Wartmann M, Cella N, Hofer P, Groner B, Liu X, Hennighausen L, Hynes NE: Lactogenic hormone activation of Stat5 and transcription of the beta-casein gene in mammary epithelial cells is independent of p42 ERK2 mitogen-activated protein kinase activity. *J. Biol. Chem.*, 271: 31863-8, 1996.

Watson CJ, Miller WR: Elevated levels of members of the STAT family of transcription factors in breast carcinoma nuclear extracts. *Br. J. Cancer*, 71: 840-4, 1995.

Watson CJ, Burdon TG: Prolactin signal transduction mechanisms in the mammary gland: the role of the Jak/Stat pathway. *Rev. Reprod.*, 1: 1-5, 1996.

Waxman DJ, Ram PA, Park SH, Choi HK: Intermittent plasma growth hormone triggers tyrosine phosphorylation and nuclear translocation of a liver-expressed, Stat 5-related DNA binding protein. Proposed role as an intracellular regulator of male-specific liver gene transcription. *J. Biol. Chem.*, 270: 13262-70, 1995.

Welsh CW, Nagasawa H: Prolactin and murine mammary tumorigenesis: a review. *Cancer Res.*, 37: 951-63, 1977.

Wen Z, Zhong Z, Darnell JE: Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. *Cell*, 82: 241-50, 1995.

Wenger CR, Beardslee S, Owens MA, et al.: DNA ploidy, S-phase, and steroid receptors in more than 127,000 breast cancer patients. *Breast Cancer Res. Treat.*, 28: 9-20, 1993.

Wennbo H, Gebre-Medhin M, Gritli-Linde A, Ohlsson C, Isaksson

OGP, Tornell J: Activation of the prolactin receptor but not the growth hormone receptor is important for induction of mammary tumors in transgenic mice. *J. Clin. Invest.*, 100: 1-8, 1997.

Wilson KC, Finbloom DS: Interferon gamma rapidly induces in human monocytes a DNA-binding factor that recognizes the gamma response region within the promoter of the gene for the high-affinity Fc gamma receptor. *Proc. Natl. Acad. Sci., USA.*, 89:11964-11968, 1992.

Wooster R, Neuhausen SL, Mangion J, et al.: Localization of a breast cancer susceptibility gene, BRCA2, to chromosome 13q12-13. *Science*, 265: 2088-2090, 1994.

Yamashita H, Xu J, Erwin RA, Farrar WL, Kirken RA, Rui HR: Phosphorylation of proline-juxtaposed serine residues S725 of Stat5a and S730 of Stat5b is not critical for prolactin-induced transactivation of a beta-casein reporter gene in COS-7 cells. Submitted for publication.

Yoshimura A, Longmore G, Lodish HF: Point mutations in the exoplasmic domain of the erythropoietin receptor resulting in

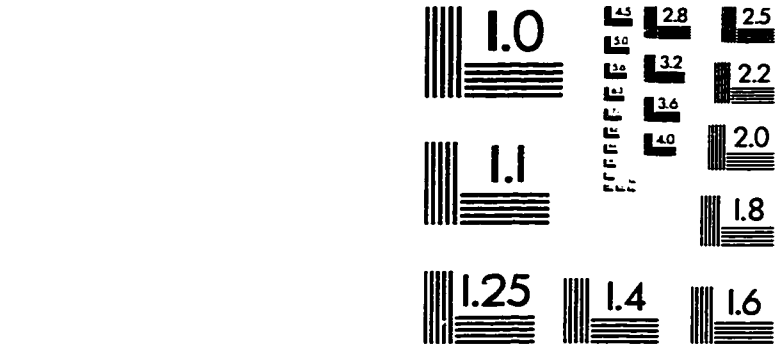
hormone-independent activation and tumorgenicity. *Nature*, 348: 647-9, 1990.

Yoshimura A, Zimmers T, Neumann D, Longmore G, Yoshimura Y, Lodish HF: Mutations in the Trp-Ser-X-Trp-Ser motif of the erythropoietin receptor abolish processing, ligand binding, and activation of the receptor. *J. Biol. Chem.*, 267: 11619-25, 1992.

Yu-Lee LY, Hrachovy JA, Stevens AM, Schwarz LA: Interferon regulatory factor 1 is an immediate-early gene under transcriptional regulation by prolactin in Nb2 T cells. *Mol. Cell. Biol.*, 10: 3087-94, 1990.

Zumoff B: Hormonal profiles in women with breast cancer. *Obstet. Gynecol. Clin. North Am.*, 21: 751-72, 1994.

IMAGE EVALUATION TEST TARGET (QA-3)



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